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C. M. WILSON

amounts of such a relatively harmless substance as sterilised indian ink may produce fatal results in young rabbits.

Quite recently Oliver (1922) has drawn attention to the occurrence of a spontaneous chronic meningo-encephalitis in rabbits. The lesions observed consist of an infiltration of the meninges and of the vascular lymph-sheaths with round cells, chiefly lymphocytes and a few plasma-cells. No micro-organisms were demonstrable in the brain. Oliver draws attention to the similarity of the lesion to that found in man in *encephalitis lethargica*. These infections in rabbits may be related to the spontaneous encephalitis of horses (Borna's disease), which recently has been shown to be transmissible to rabbits by experimental inoculation (Moussu, 1922).

Secondly, other filter-passing viruses tend to produce a somewhat similar histological change, viz. poliomyelitis, rabies, vaccinia, nervous herpes. Poliomyelitis, however, can only be transmitted to rabbits with the greatest difficulty, and when accomplished, many polynuclear leucocytes are to be found in the infiltrations. Rabies is an unlikely possibility, and the previous history of the case from which the material was derived should be sufficient. Vaccinia virus, as was recently shown by Levaditi and Nicolau (1921), can produce changes in the rabbit's brain almost indistinguishable from those of encephalitis, but I have found that although a primary infection in the cerebrum is obtained, it is only with the greatest difficulty that the virus can be carried on in series by brain inoculations alone. The cellular infiltration is more meningeal than perivascular and contains many large mononuclear cells; on the other hand, in *encephalitis lethargica* the cellular infiltration consists mainly of lymphocytes and glial cells.

CONCLUSIONS.

From the above observations it would appear that rabbit inoculations are of the greatest value in confirming a diagnosis of *encephalitis lethargica*.

Emulsions made of the basal nuclei give in most cases a positive result in the rabbit, while cerebrospinal fluid was only positive in one out of four cases.

It seems probable, from the findings of Kling, that a higher percentage of positive findings might be obtained with cerebrospinal fluid if the rabbits which show no signs after inoculation were killed 10 to 20 days later and the brain examined histologically.

In conclusion, it is necessary to impress on the observer that the important features of the lesion are (1) the site, (2) the nature of the cells, and (3) the presence of small foci of cells in the brain substance.

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OF

EXPERIMENTAL PATHOLOGY

ON THE BEHAVIOUR OF PLATELETS IN VITAMIN A DEFICIENCY AND ON THE TECHNIQUE OF COUNTING THEM.

W. CRAMER, A. H. DREW AND J. C. MOTTRAM.

From the Laboratories of the Imperial Cancer Research Fund, and from the Radium Institute, London.

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In a series of papers we have shown (1921, 1922) that there is an essential similarity between the effects of radiations from X rays and radium on the one hand, and, on the other, of submitting animals to a diet deficient in vitamins. Thus a diet free from vitamin B produces atrophy of the lymphoid tissue throughout the body and a lymphopenia, just as radiations do. This is associated in both cases with marasmus, fall of temperature and eventually death in the absence of infections or other obvious lesions. A diet free from vitamin A was found to produce a thrombopenia, and when as the result of this finding the behaviour of blood-platelets in radiated rats was studied, it was found that the same lesion of thrombopenia could be produced if a sufficiently large dose of radium was applied. This thrombopenia was associated with the same general effects in both conditions, namely, an anæmia and a diminished resistance to infection. Recently yet another lesion has been found to be present in both conditions, namely, an atrophy of the intestinal epithelium. This will be dealt with in detail in separate communications. It is sufficient to state here that probably this lesion is the primary lesion which leads to invasion of the blood-stream by the intestinal bacteria, which are there agglutinated by the blood-platelets. This, by acting as a constant drain on the blood-platelets, exhausts their supply, and may thus account for the thrombopenia. In any case whatever the explanation may be, the generalisation that vitamin deficiency and radiations produce the same general effects has so far proved to hold good.

The occurrence of a thrombopenia after radiation has been confirmed by the independent work of Fabricius-Möller (1922), who studied the effects of X rays on guinea-pigs. He obtained a degree of thrombopenia even more profound than we could produce in rats with radium, and found that this intense thrombopenia was accompanied by hæmorrhages in different organs. And Lacassagne and his collaborators (1922) produced in newborn rabbits a condition of purpura hæmorrhagica with almost complete disappearance of platelets by applying X rays to the uterus of the pregnant mother shortly before birth.

The behaviour of the blood-platelets in vitamin A deficiency has been studied recently by Bedson and Zilva (1923) in rats. They conclude from their observations that there is a slight diminution of the blood-platelets in that condition. That diminution is, however, much less than the thrombopenia obtained by us. Thus while the diminution in our advanced cases was never less than 50 per cent. of the normal number and sometimes exceeded 75 per cent., Bedson and Zilva find that the diminution in their rats even when they exhibit xerophthalmia amounts maximally only to 20 per cent. In absolute figures, our normal rats have an average count of about 800,000 platelets per c.mm., and this falls in vitamin A deficiency to 400,000, 300,000 or even 200,000. The counts of Bedson and Zilva give for normal rats roughly 1,000,000 per c.mm., and for vitamin A deficiency rats a maximal fall to only 800,000. Bedson and Zilva state that they are unable to account for the discrepancy between our figures and theirs. They do not disclose the fact that in a small number of animals comparative counts were made by them and by us on the same animal. In normal rats these counts gave a reasonably close agreement. But in rats suffering from vitamin A deficiency the number of platelets found by Bedson and Zilva widely exceeded our counts, sometimes by nearly 100 per cent. And while our counts always indicated the presence of a marked thrombopenia, even in Bedson and Zilva's rats, their counts indicated only a slight thrombopenia or no thrombopenia at all, even in our rats. These facts are of importance, for they prove that the discrepancy must be due to differences in technique. It definitely eliminates the possibility that thrombopenia is an accidental phenomenon which may occur occasionally.

We shall demonstrate in this paper that Bedson and Zilva on their own showing used such an optical technique as would make an accurate counting of blood-platelets impossible, especially in such pathological conditions as vitamin A deficiency, where the blood contains a number of bodies similar in size to blood-platelets, but obviously different if examined with a microscope giving the highest possible resolution.

Owing to faulty microscopic technique errors have been made in the past by grouping together as " blood-platelets " all small bodies floating in plasma and being neither red corpuscles nor leucocytes. Thus faulty microscopic technique is responsible for the extraordinary mistake of denying altogether the existence of blood-platelets as preformed living elements of the blood. Some of the older histologists who carefully studied the behaviour and structure of blood-platelets in great detail have pointed out the error of confusing platelets with other bodies, and they insisted that blood-platelets must be identified by their characteristic structure. Under a good lens-system

living unstained rat platelets appear in front view as circular, sharply outlined discs, having an average diameter of 2 to 3μ, and containing minute highly refractile granules grouped towards the centre. Seen edgeways a platelet is a very narrow concavo-convex disc with a slight thickening in the centre. In vitamin A deficiency the blood is full of bodies which do not have the structure of blood-platelets. Some of these bodies can be recognised as bacteria, others are undefinable and may represent lipoid globules or protoplasmic *débris*. This greatly adds to the difficulty of counting in that condition because every blood-platelet has to be identified by its structure. Since details of structure of blood-platelets are very near the limit of microscopic vision and have to be recognised in the living unstained cell, their identification requires the highest possible power of resolution a microscope can give. We find no mention of this difficulty of distinguishing blood-platelets from other bodies in the paper by Bedson and Zilva, and it is in fact impossible to do so with any degree of certainty with the optical arrangement used by these authors. We can say, however, that in the two rats which we received from them such bodies were present in large numbers. Since this question of microscopic optics appears to be so little appreciated, it is necessary to go into the matter more fully.

TABLE.

No. of rat.	Number of red cells.	Number of platelets.	Duration of vitamin A deficiency.	Growth.	Eye symptoms.	
442	9,880,000	340,000	8 weeks.	Slowed	None	
443	8,760,000	415,000	8 „	„	„	
444	7,480,000	240,000	12 „	Arrested	„	
445	6,440,000	250,000	4 „	„	Xeropthalmia	
446	10,040,000	1,070,000	Before deficiency	Normal	—	Exposure to mercury vapour lamp from 1st to 9th week.
	8,080,000	705,000	4 weeks	Slowed	None	
	8,920,000	706,000	7 „	„	„	
	7,400,000	297,000	16 „	Arrested	„	
447	9,400,000	610,000	1 week	Normal	„	Mercury vapour lamp from 4th to 7th week.
	7,720,000	380,000	4 weeks	Arrested	„	
	9,040,000	460,000	7 „	„	Xeropthalmia	
448	9,040,000	650,000	1 week	„	None	
	7,320,000	575,000	4 weeks	„	„	
529	9,400,000	610,000	2 „	„	„	
531	8,920,000	890,000	1 week	Normal	„	
	8,800,000	546,000	7 weeks	Arrested	„	

As regards the phenomenon itself which is in question, we have since the publication of our first paper made further observations on the number of platelets in rats on a diet deficient in vitamin A in order to study the effect of light on the organism. As the following table shows, we never failed to obtain a deep thrombopenia corresponding in degree to that recorded in our first paper. We stated there that in our stock of rats the normal count lies between 9 million and 10 million red cells and between 700,000 and 900,000 platelets. It should be noted that most of the counts given in this paper

were made while the animals were still free from obvious infections and had not yet reached the final stage of vitamin A deficiency, because it was desired to study the effect of light on the thrombopenia in different stages of the deficiency. Two rats are included in this table which had been treated with ultra-violet light. No. 446 received light throughout the first nine weeks of the experiments, and shows a greatly delayed thrombopenia as compared with rats Nos. 447 and 448 of the same litter. Rat 447 received treatment with ultra-violet light after a deep thrombopenia had developed, with the result that the fall in the number of platelets was arrested. We intend to publish detailed observations on the action of light in a separate paper.

THE NECESSITY FOR USING A PERFECT OPTICAL SYSTEM IN THE COUNTING OF PLATELETS.

In ordinary histological or bacteriological work differentiation of structure is obtained by means of staining, which reveals the various cell structures by colour contrast. For this purpose the old-fashioned Abbe condenser generally suffices, although every scientific microscopist knows that it is defective in many respects, and that in particular it is incapable of giving perfect resolution, no matter how perfect the objective is. It has, however, the advantage of being fool-proof, and enables the routine worker who, as Nelson has pointed out, has no knowledge of the use of a proper aplanatic cone in his illuminating system,* or of the necessity of tube length correction, to obtain reasonably good results. But when one examines living unstained cells—as is necessary in the counting of platelets—details of structure can be recognised only through difference in the refractive index of the different cell constituents. This requires the highest resolution of which the microscope is capable, especially when one is dealing with such minute structures as the granules in platelets, which are near the limit of microscopic vision. Then the condensing system becomes all-important.

It must be clearly understood that resolution and magnification are two entirely different things, and that no amount of magnification can compensate for imperfect resolution. This elementary principle of microscopic optics is not acted upon by Bedson and Zilva, for when they wish to improve their optical system they substitute a 2 mm. oil-immersion lens and a high-power ocular for a 4 mm. dry lens and a low-power ocular, while retaining their Abbe condenser. By doing so they merely magnify an imperfectly resolved image, but, as will be shown below, they do not to any extent improve the resolution of the image. Resolution depends on the optical angle of the cone of light illuminating the object and the apical angle of the cone of light entering the objective. The wider the angles the greater the resolution. A measure of the width of these angles is given by the "numerical aperture" (N.A.) of the condenser and of the objective. In scientific microscopy the resolving

* All optical systems in the microscope are computed on the assumption that light enters the condenser with a plane homogeneous wave front and with the rays parallel to the optical axis of the system, that it is transformed by the condenser into light having a spherical homogeneous front with the object at the centre of the sphere, and is transformed again by the objective into light having a plane homogeneous wave front. The perfection of the image depends on the extent to which this condition is fulfilled.

power of any optical system is therefore expressed by the mean of the actual working N.A. of the condenser and of the objective. We say advisedly "the actual working N.A.," because the nominal N.A. of the condenser given in the catalogues does not necessarily represent the actual working N.A. Bedson and Zilva in describing their technique state: "An Abbe condenser (N.A. 1·3) was used." Now the Abbe condenser transmits a cone of light having a N.A. of 1·3 only when a layer of oil is interposed between condenser and the slide. But since, as Dallinger pointed out, less than half of that cone is aplanatic, only the aplanatic portion of the cone takes part in the formation of the image. The utilised fraction of the cone of light transmitted by an Abbe condenser is therefore reduced from 1·3 to 0·45, which makes this condenser useless for critical microscopy. Thus a 2 mm. lens of N.A. = 1·30 with an Abbe condenser gives an effective N.A. of $\frac{0{\cdot}45 + 1{\cdot}30}{2} = 0{\cdot}87$. That is the optical system used by Bedson and Zilva. Our system consisted of 2 mm. apochromat objective of N.A. = 1·40 with a first-class apochromatic condenser which can transmit an aplanatic cone having the N.A. = 1·35. Our system gives therefore an effective N.A. of $\frac{1{\cdot}40 + 1{\cdot}35}{2} = 1{\cdot}37$—an increase in the resolving power of over 50 per cent. as compared with the imperfect system of Bedson and Zilva. In fact Bedson and Zilva could have obtained a better resolution with a dry apochromatic 4 mm. lens of N.A. = 0·95 if they had combined it with a first-class condenser transmitting an aplanatic cone of N.A. = 0·90. Such a system would give them a N.A. = 0·92 instead of the value of 0·87 for their optical system with an oil-immersion lens. To get better definition with the Abbe condenser they recommend closing down the iris diaphragm, thus still further reducing the width of the aplanatic cone upon which resolution depends.

In order to demonstrate the fallacies introduced by counting platelets with an improperly worked optical system, we have made counts of the blood-platelets of vitamin A deficient rats both with an improperly worked optical system and with a properly worked system. The latter consisted of a 2 mm. apochromat objective, N.A. = 1·40, with a holoscopic oil-immersion condenser having a nominal N.A. = 1·40 and giving an actual cone of N.A. = 1·35. The imperfect system consisted of a 2 mm. semi-apochromatic oil-immersion objective, N.A. = 1·30, and an Abbe condenser, and corresponds to the system used by Bedson and Zilva.

The following figures were obtained:

	Perfect optical system. N.A. = 1·37.	Imperfect optical system. N.A. = 0·87, as used by Bedson and Zilva.
Rat 1	250,000	610,000
Rat 2	575,000	869,000
Rat 3	380,000	711,000

With the imperfect optical system as used by Bedson and Zilva the figures are always higher, and in fact approximate to those given by these authors, and if we had used such a defective system we would also have failed to detect the intense thrombopenia which actually exists. We had the same experience

when in a few cases counts were made by us on rats in A deficiency which had been counted previously by Bedson and Zilva, or when, conversely, Bedson and Zilva counted rats in A deficiency which had been counted previously by us. In every case the counts by Bedson and Zilva were much higher than our counts—sometimes twice as high. With the blood of normal animals these discrepancies are not so marked, because normal blood contains few bodies which could be mistaken for platelets.

These facts speak for themselves so far as the results of Bedson and Zilva are concerned. Speaking generally, they mean that when an imperfect optical system is used in counting blood-platelets in vitamin A deficiency, a large number of bodies are counted as blood-platelets which, when viewed with a better optical system, are recognised as not being blood-platelets.

THE TECHNIQUE OF PLATELET COUNTING.

In view of the errors resulting from imperfect microscopic technique, it may be useful to give a more detailed account of the method of platelet counting than we gave in our first paper.

The necessary requisites are:

(1) A suitable diluting fluid.
(2) A Thoma-Zeiss hæmacytometer.
(3) A first-class microscope stand provided with a 2 mm. oil-immersion apochromat objective and an oil-immersion condenser, giving a solid cone of illumination equal to at least three-fourths of the N.A. of the objective.
(4) Suitable screens to give a ruling in order to facilitate counting.

The diluting fluid we have used and found suitable is Toisson's fluid. It must be freshly made and filtered before use. The rat is deeply anæsthetised with ether, the tail immersed for a few seconds in a vessel containing warm water at about 45° C. and then dried with cotton-wool. The tail is then completely immersed in a watch-glass or evaporating dish containing Toisson's fluid. It is important that the tail must be thoroughly wetted with Toisson's fluid. The tail is now cut under the fluid about 1 or 2 in. from the end and blood allowed to flow into the fluid. It is essential that a free flow of blood is obtained. After allowing the blood to flow freely for a few seconds, the end of the tail is quickly transferred to a watch-glass containing about 2 or 3 c.c. of Toisson's fluid, so that the end of the tail is completely immersed in the fluid. Blood is allowed to flow until a dilution suitable for counting is obtained. We have found it best to have a dilution so as to get about thirty red blood-cells in one field. After a little practice it can readily be seen when such a dilution is reached. The tail is then removed from the saline fluid, dried, and the red blood-cell pipette of the hæmacytometer filled in the usual way for a red-cell count.

The diluted blood is now thoroughly mixed with a platinum loop, and a standard drop placed on a glass slide previously cleaned with chromic acid and stored in alcohol. A No. 2 cover-glass, previously cleaned, is placed on the drop, which should spread out as a single cell-layer if slide and cover-glass have been properly cleaned. The preparation is now ringed round with

paraffin wax and put aside to allow the blood-cells to settle. In the meantime a red cell-count is made in the usual way. When this has been completed the slide for the platelet-count is placed under the microscope.

For the reasons given in the preceding paper the optical system used must fulfil certain conditions. The condenser must be an oil-immersion condenser, giving a cone proportionate to the N.A. of the objective, so that the cone of illumination used is at least three-fourths of the N.A. of the objective. This can be done with any of the modern oil-immersion condensers. The condenser must be accurately focussed and centred, so that the image of the illuminant is seen in the same plane as the object. The tube-length must be accurately adjusted. The eye-piece is chosen so as to give a magnification of about 1200 diameters.

In order to facilitate the counting some form of ruling is necessary. This may consist of a glass circle ruled in millimetre squares, which is dropped into the ocular. Or a modification of the "ghost micrometer," as devised by Prof. Dixon, F.R.S., may be employed, and has been found very convenient.* It consists of a glass slide ruled into square millimetres and fixed in front of the illuminant in the plane of the lamp iris diaphragm. This slide now takes the place of the source of the light and an image of the squares is found focussed on the object. As already stated, the most suitable dilution for counting is one where about thirty red cells and in a normal rat about three platelets are seen in one field. The number of red cells in the various fields is now counted along with the platelets, and thus the ratio of red cells to platelets is obtained. Each individual platelet must be identified by its structure. The distribution of the red cells in different fields and the proportion of red cells to platelets in each field should be even. If the proportion is very uneven another preparation should be made.

With a good preparation it is sufficient to count thirty to forty platelets. This involves the counting of 300 to 400 red cells in normal rats and of 600 to 800 red cells in vitamin A deficient rats. The statement of Bedson and Zilva that they counted 100 platelets must be viewed in the light of the demonstration given in the preceding section, that in the vitamin-deficient rats more than half the bodies which with a defective optical system are apt to be counted as platelets are not blood-platelets at all.

It may be added that in our experiments the factor of personal bias was completely excluded. The person who counted the platelets did not know what the condition of the animal was, and as a rule animals in a state of vitamin deficiency and normal animals or animals recovering from a state of vitamin A deficiency were subjected to examination at the same time.

SUMMARY.

The constant occurrence of a deep thrombopenia in vitamin A deficiency in rats has been confirmed by us by further observations. A detailed account is given of the technique of platelet counting, with special reference to the microscopic technique required to obtain reliable results. It is shown that with a faulty optical system the platelet-count in vitamin A deficiency gives

* Dixon's "ghost micrometer" is supplied by T. H. Mason, Dublin; our modification may be obtained from James Swift & Son, Tottenham Court Rd., London.

figures which greatly exceed, sometimes to the extent of 100 per cent., those obtained with a correct optical system giving the highest possible resolution This is due to the fact that in vitamin A deficiency a number of bodies other than platelets but of similar dimensions appear in the blood and are mistaken for platelets when a faulty microscopic technique is employed. The optical system used by Bedson and Zilva, who, in repeating our observations, found only a slight thrombopenia in vitamin A deficiency, is a faulty one, and the platelet counts obtained by them greatly exceed the true counts obtained with a correct optical system. That the results of Bedson and Zilva are due to their microscopic technique and not to any difference in the reaction of their rats to vitamin A deficiency follows from the results obtained when counts were made on the same animal in three cases of vitamin A deficiency by them and by us. In every case their figures greatly exceeded ours.

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A SUGGESTED SUBSTITUTE FOR CANADA BALSAM AS A MOUNTING MEDIUM.*

H. W. SOUTHGATE, M.B., B.Sc.

From the University, Sheffield.

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THE effect of Canada balsam on the more delicate stains is known only too well to those interested in histology. In my opinion this is due mainly to the 63 per cent. of higher organic acids contained in this balsam.

In 1920 and the early part of 1921 efforts were made, but in vain, to obtain a neutralised non-crystalline solution of Canada balsam in xylol, by neutralising with various inorganic and organic bases.

The literature concerning other oleoresins was therefore consulted with a view to finding a substitute for Canada balsam, and it was found that a species of Elemi—that from Yucatan—contained no organic acids, but 60–70 per cent. neutral amorphous resenes besides volatile oil and amyrin. Through the offices of a mutual friend, Dr. Tschirch sent me the remainder of the original

* This note is an abstract of a fuller paper to appear in the *Journal of the Royal Microscopical Society*, and is published through the courtesy of the Editors of the latter.

sample of Yucatan Elemi, his analysis of which had drawn my attention to its possibilities as a substitute for Canada balsam.

The refractive index of a 60 per cent. solution of some of this in benzene was compared with that of a similar strength solution of natural (*i. e.* undried) Canada balsam in the same solvent, and gave—

60 per cent. Elemi solution	1·5146
60 „ Canada balsam	1·5073

for the hydrogen red line.

Solutions of this Elemi also dried and set quickly.

Liver sections from a case of malignant malaria were stained with Giemsa and put up in a solution of the oleoresin in xylol. The colours were maintained but a difficulty rapidly appeared, *i. e.* the crystallising out of the amyrin compounds under the cover-slip. Efforts were made to remove these compounds by recognised methods but I was not able to remove sufficient to prevent some crystallisation taking place, and the investigation was discontinued in the summer of 1921.

Recently, however, I brought the possibility of this Elemi (provided a more complete removal of the amyrins could be effected) to the notice of a friend (Mr. P. P. Laidlaw), and he informs me that the following method removes some 25 per cent. of crystalline compounds (a bigger yield than I obtained), and that some slides mounted now for over three months in this medium show no trace of crystallisation, and, it is presumed, will not now.

He removes the amyrins by the following simple method:

200 grams of crude hard Yucatan Elemi are digested in the cold with 200 c.c. of industrial spirit (not methylated spirit) till all the lumps are broken up. The resene and the essential oil dissolve readily; the bulk of the amyrin crystals and the *débris* remain suspended, forming a mixture of creamy appearance. The amyrin crystals and the *débris* are filtered off at the water-pump, the filter cake being well pressed out. The filtrate is then evaporated down in the steam oven in shallow dishes, and when dry dissolved in *dry* xylol to the desired consistency for mounting.

Yucatan Elemi with the amyrins removed by the above method is therefore recommended as a substitute for Canada balsam as a mounting medium for the following reasons:

(1) It contains no organic acids but consists practically of neutral resenes.

(2) Complete solubility in alcohol, xylol, benzene, etc.

(3) A comparable refractive index.

(4) Its good drying and setting properties.

(5) Some sections stained with Giemsa's stain two years previously have the colours still unimpaired, mounted in this medium.

(6) Its relative cheapness: the crude mountant can be purchased from The British Drug Houses Ltd. for about 2*s.* 6*d.* a lb., and this should yield about 60 per cent. of neutral resene.

Finally it may be stated that Mr. C. Dobell reports very favourably on this Elemi solution in dry xylol as a mountant for the Romanowsky stains, methylene blue, etc. Methylblau, however, he informs me, is an exception.

My indebtedness to Mr. P. P. Laidlaw is obvious, and I would express my thanks to Mr. C. Dobell also for his kindly interest and criticisms.

GROWTH AND DIFFERENTIATION IN TISSUE CULTURES.

A. H. DREW, D.Sc.

Imperial Cancer Research Fund, London.

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THE study of pure cultures of cells *in vitro* permits an insight into the relations of cells of different kinds to each other, and leads to an analysis of the factors which determine their behaviour in the body with the ultimate possibility of the experimental control of the processes involved. Differentiation is one of the most obvious of these processes, and the observations to be described here indicate some of the factors which influence it, and reveal its reversible character. In an investigation of this kind technical details inevitably play an important part, and it will be necessary to describe the modifications which have been adopted as a result of extended trials since the publication of the previous communication in this Journal (Drew, 1922).

It has been shown already that cultures of both embryonic and adult tissues as well as tumours may be grown in a saline medium for an indefinite period so long as an embryonic extract is present as well. Growth does not occur without such an extract in the case of adult tissues, whilst in tumours and embryonic tissues generally a temporary growth takes place. The substance or substances in embryo extract responsible for maintaining growth are unknown, but the activity diminishes on keeping in the ice safe (4°–8° C.), and in two weeks is no longer of usable strength. They are destroyed by heating at 60° C. for 15 minutes. Filtration through a Chamberland F candle definitely reduces the strength of the extracts; filtration through a Berkefeld candle, however, leaves this property unimpaired, and gives a clear, nearly colourless sterile filtrate which may be kept for a much greater period without undergoing change. The method of preparation is as follows: Young rat or mouse embryos are cut up into coarse pieces with scissors in a sterile Petri dish. The fragments are thoroughly washed with several changes of the saline culture medium to remove traces of blood and are then finely minced. (In the preparation of all tissues for *in vitro* culture, blood must be got rid of as completely as possible, as if present in more than traces, it appears to inhibit growth.) The pulp so obtained is transferred to a large sterile tube and is frozen and thawed three times to disintegrate the cells and liberate the juices. An equal volume of the saline fluid is added to the emulsion, and is incubated for an hour at 37° C., the mixture being shaken at intervals. At the end of an hour the tube is centrifuged at high speed and the supernatant fluid pipetted off. It is then filtered through a Berkefeld filter and is stored in tubes in the ice chest.

It has been found best to use a final fluid made up as follows:

Saline solution . . .	3 parts.
Embryo extract . . .	1 part.
Rat serum . . .	1 „

The addition of the rat serum is not necessary for growth, which occurs perfectly well without it, but it makes the fluid more viscid, prevents the drop from spreading over the entire surface, and tends to stick the fragment of tissue to the cover-glass. To ensure adhesion the hanging-drop preparation is inverted and left in the incubator for about 6 hours, or indeed throughout incubation. It is only turned over when it is desired to examine the culture through the cover-glass, under the microscope.

Extract prepared in a similar manner from adult tissues gave little or no growth, but if very much stronger extracts were made by reducing the volume of saline added to about one-tenth they were found to be capable of replacing embryo extract to a certain extent. Such extracts were more potent when prepared from young rats or mice, indeed extracts from old animals appeared to exert an inhibitory effect, inducing early degeneration in the young growing cells. This phenomenon has already been noticed by Carrel and Ebeling (1921) to occur in plasma cultures, the plasma of an old animal retarding growth. Tumour extracts were found to be somewhat erratic, but could replace embryo extract with fair success, if used in sufficient concentration, to maintain growth in normal tissues. When tested with cultures of tumours they generally proved unsatisfactory, inducing early degeneration. This effect is probably due to toxic bodies, as it has already been shown that cultures of actively growing tumour cells appear to produce a substance capable of inhibiting growth in tumour cultures.* A yeast extract, rich in vitamin B, was tried but was found to be perfectly inert, and could in no way replace embryo extract. A pea flour extract likewise was quite useless. Apparently both adult tissue extracts and tumour extracts contain the substance or substances present in embryo extract, but in the case of tumours this is masked by the toxic bodies, whilst old tissues contain an inhibitory substance.

I have already shown that differentiation of the cells in a culture depends upon the presence or absence of connective tissue, but the best demonstration of this fact is seen when one grows pure cultures of cells, *e. g.* kidney or skin, and then adds connective-tissue elements. Pure cultures may be obtained with little difficulty by the mercury drop method used for obtaining single cell bacterial cultures. A fragment of embryonic kidney is planted out in the fluid medium and incubated at 37° C. The fragment is examined daily, and whether it shows commencing growth or not, fresh medium is supplied each day. At the end of four or five days, sometimes earlier, the kidney cells will be found growing out as elongated processes which ultimately form a sheet of undifferentiated tissue. The processes in many cases are quite free from accompanying connective tissue. A growth is selected with processes free from other cells, and a globule of mercury is carefully placed on the slide and

* The growth-activating substance from autolysed normal tissues, which is mentioned on a subsequent page, has a similar action when applied continuously to growing normal tissues, but it seems probable that the toxic action of extracts of tumour cultures is not identical.

pushed over a projecting process of parenchyma cells so as to cover it. The slide is then removed from the microscope and is exposed to a quartz mercury vapour lamp for ten minutes, care being taken to avoid heating. Fresh culture medium

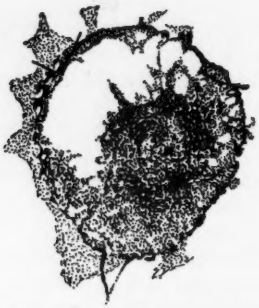

FIG. 4.—Mouse carcinoma 206 in culture in saline medium + embryonic extract. Low power view. See Plate-fig. 4*a*.

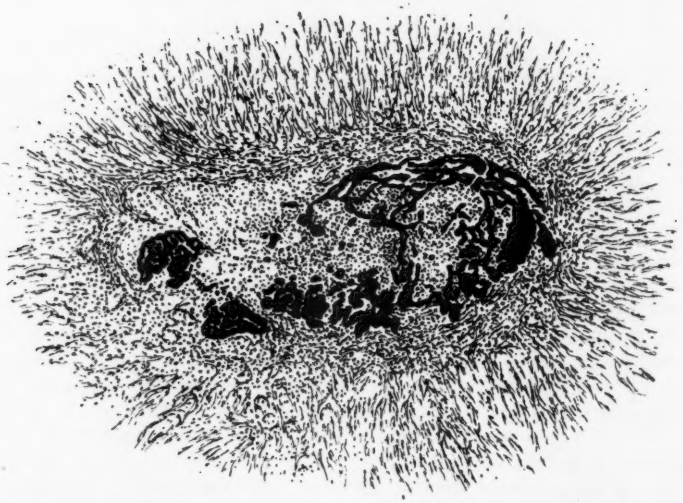

FIG. 5.—Mouse carcinoma 206 in culture with connective-tissue elements. Note acinus formation. The whole growth strongly resembles normal mouse mamma.

is added and the slide returned to the incubator. The cells unshielded by the mercury globule are killed and growth now proceeds from the isolated process. The culture is continued till a large sheet of undifferentiated kidney cells is obtained (Fig. 1, and, in the case of mammary tissue, Fig. 2), when

a small fragment of a connective-tissue culture is added and incubation continued. In a certain proportion of cases the connective tissue may fail to grow, but in those cultures where it does so differentiation sets in in about two to three days, and a culture is obtained containing free connective-tissue cells, undifferentiated kidney cells and kidney cells forming typical tubules (Figs. 3 and 3*a*). The same process is shown very well with tumours. For example, an alveolar mammary carcinoma, 206, tends in artificial culture to give pure parenchyma growths, as a sheet of undifferentiated cells (Figs. 4 and 4*a*), without having recourse to exposure to ultra-violet rays. When such a culture is obtained, either by aid of the ultra-violet light or from cultures growing pure from the start, connective-tissue cells may be added either from a culture or from a fresh piece of tissue planted direct. Fig. 5 illustrates the type of growth obtained. Where the tumour cells come into contact with the connective tissue they form acini, the whole culture strongly resembling normal mouse mamma (Figs. 6 and 7). This experiment shows that the undifferentiated condition of the parenchyma of this tumour is not an anaplasia in v. Hansemann's sense (*i. e.* a permanent irreversible loss of differentiation), but merely a temporary growth form which can differentiate out in a suitable environment. The connective-tissue elements may be derived from any organ. For example, connective tissue derived from heart will cause the formation of acini in a mammary carcinoma or the formation of tubules in cultures of kidney. That there exists a delicate balance between the two types of cells is clearly shown in continued culture, for if such a culture, say of kidney, which has had tubule formation induced by the addition of connective-tissue elements to it, be continued, it is found that gradually only one or other of the types of cell will persist, and ultimately either a culture of connective tissue or of pure kidney cells will be obtained again, when the cells grow as a sheet of undifferentiated tissue as before. In most cases the connective-tissue cells grow more rapidly than the parenchyma, and ultimately tend to overgrow it. In the case of mixed cultures of tumour and connective tissue artificially added, the tumour cells rapidly degenerate unless very frequent subculture is performed and one ultimately obtains a culture of connective tissue only. Cultures of carcinoma with added stroma exhibiting differentiation reproduce the original type of tumour on reinoculation into mice.

Attempts to inhibit the growth of tumours in mice by means of the toxic products elaborated *in vitro* have not proved successful. Whatever the toxic substance is that is formed in culture, it is apparently easily destroyed in the body and fails to influence the growth of the tumour. Cultures of normal tissues grown for many generations outside the body show no tendency to grow when inoculated into animals but rapidly disappear.

We have found it preferable when removing normal or malignant tissues for cultures to store them in the ice-chest when immediate culture is not possible. Such cold storage, provided the tissues are aseptic, appears to do no harm, growths having been obtained after as long a period as thirteen days. Comparatively slight alterations in the incubation temperature appear to have a considerable effect on the cultures. For example, heart cultures incubated at 40° C. give growths of connective-tissue cells only, the heart muscle cells showing fatty changes and rapidly dying out. Mammary carcinoma incubated

at the same temperature gave pure connective-tissue growths after two generations, the tumour cells degenerating. Kidney, on the other hand, appeared to be little influenced by this temperature, the same proportion of kidney growths resulting as at 37° C. Tumours when grown on slides completely immersed in the culture medium are interesting from the fact that portions become detached and grow on distant parts of the slide forming what may be termed artificial metastases: such a growth is illustrated in Fig. 8.

An interesting fact is brought out in comparing the malignancy with the degree of growth *in vitro* of a series of tumours of varying degrees of malignancy, for example 37 S, 63, 206, Jensen, 630, 27. These are arranged in Table I in order of their rapidity of growth in the mouse. *In vitro* cultures of these tumours show growth which may be represented by the *plus* signs in the table:

TABLE I.

Rapidly growing spindle-cell sarcoma 37 S	+ + +
Rapidly growing alveolar mammary carcinoma 63	+ + +
Mammary carcinoma, rapidly growing, but with much absorption 206	+ +
Variably growing alveolar mammary carcinoma Jensen	+
Squamous cell carcinoma 630	slight + after lag
Adeno-carcinoma of mamma 27	„ „

Where growth is obtained at all from 630 and 27 it closely resembles that of adult tissues, being small in amount at first and always exhibiting the lag which seems characteristic of adult mouse and rat tissues.

The ease with which the several factors, which may be supposed to act in growth and repair, can be separately varied in *in vitro* culture has already been noted. Striking differences are produced in the cultures of adult tissues by adding to the culture medium an extract of autolysed adult tissue. Adult kidney was ground up with sterile sand and some of the saline medium; the resulting emulsion was incubated for one hour at 37°C., and was then filtered through a Berkefeld candle. Two series (A and B) of primary cultures of heart and kidney obtained from a full-grown mouse were put up in media having the following composition:

A. Saline solution	3 parts.
Embryo extract	2 „
Rat serum	1 part.
B. Saline solution	3 parts.
Embryo extract	1 part.
Autolysed kidney extract	1 „
Rat serum	1 „

A dozen cultures of heart and kidney were put up in each of the solutions A and B, incubated at 37°C., and examined daily.

In the cultures without damaged tissue extract no growth occurred till the

tenth day, when a mixed growth containing connective-tissue cells and kidney and heart cells respectively was obtained from 8 of the 12 cultures (Fig. 9).

Cultures in solution B showed connective-tissue growth within twenty-four hours in all cases, but only slight growth of the parenchyma took place till the second day, when all the cultures showed a mixed growth of both kidney and connective-tissue cells (Fig. 10). It will be seen that there appears to be some substance or substances in an extract of damaged tissue capable of accelerating the growth both of connective tissue and parenchyma. Heating to 80°C. for 15 minutes had no effect on the growth-promoting substances. A feature of the growth obtained by the addition of such autolysed extracts is its explosive character—that is to say, the original fragment may disappear and fragments of rapidly growing cells be found scattered over the slide. If such growths be subcultured into fresh medium containing autolysed extract little more growth is obtained as rapid degeneration sets in. If, however, the subculture is made with the ordinary culture fluid, vigorous growth will continue and the cultures may be maintained indefinitely.

It seemed likely that tumours might contain such a growth-accelerating substance ready formed, and to test this supposition extracts of tumours were prepared by grinding up tumour cells in a mortar cooled with ice. A small quantity of the saline medium was added and the mixture centrifuged at once without incubation. On testing such extracts it was found that a very potent growth-accelerating substance was present, giving as much growth with adult tissues as did an autolysed extract of normal tissues (Figs. 11, 12, 13). The tumours tested gave varying amounts of the activating substance. Thus both carcinoma 63 and sarcoma 37 yielded extracts of great potency, and both these tumours are of a high degree of malignancy and of very rapid growth (*cf.* Table I). Carcinoma 27, on the other hand, which is of low malignancy and slow growth, yielded only small traces of the activating substances, being in fact intermediate between the very malignant tumours and normal tissue.

The activating substances in autolysed extracts of normal adult tissues and their absence in embryonic tissue extracts shows that they are undoubtedly the same agents which are met with in the phenomena of repair. Their presence in cold extracts of the malignant tumours suggests that it is by means of a continuous production of these substances that tumour growth is maintained, and constitutes a new and very striking difference between tumours and normal tissues, the former containing the growth-stimulating factors, whilst the latter require to be severely damaged before they are produced. The fact that the highly malignant and rapidly-growing tumours contain a far greater amount of such growth-activating substances than do the slowly-growing and more benign, reveals a quantitative relation which has not before been demonstrated in the analysis of tumour growth.

SUMMARY.

The substances necessary for continuous growth which are present in extracts of embryonic tissues are able to pass through a Berkefeld filter. Adult tissues and tumours appear to contain these substances in lower

concentration. Tissues and tumours when grown free from connective-tissue elements grow as undifferentiated sheets. If connective tissue be added to such growths differentiation sets in. Autolysed extracts of normal adult tissues contain a growth-activating substance which when added to cultures of normal adult cells causes very rapid proliferation. Extracts of tumours, prepared in the cold and not subjected to autolysis, also contain this substance, the tumour cells appearing to be able to form it continuously.

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CARREL, A., AND EBELING, A. H.—(1921) "Age and Multiplication of Fibroblasts," *J. Exp. Med.*, **34**, 600.

DREW, A. H.—(1922) "A Comparative Study of Normal and Malignant Tissues Grown in Artificial Culture," *Brit. J. Exp. Pathol.*, **3**, 20.

DESCRIPTION OF PLATES.

FIG. 1.—Four days' growth of embryonic mouse kidney in saline medium + embryonic extract, showing growth of parenchyma as undifferentiated sheet of cells.

FIG. 2.—Culture of normal pregnant mouse mamma in saline medium + embryonic extract. Ten days' growth, showing sheet growth of undifferentiated cells. Note formation of milk.

FIGS. 3 and 3*a*.—Portion of culture of mouse kidney to which connective-tissue elements have been added. Note tubule formation. Four days' growth. Low and high power.

FIG. 4*a*.—Mouse carcinoma 206 in culture in saline medium + embryonic extract. High-power view. Note growth as undifferentiated sheet.

FIG. 6.—Mouse carcinoma 206 grown with embryonic rat heart. Five days' growth. Note formation of acini.

FIG. 7.—High-power view of portion of culture shown in fig. 6.

FIG. 8.—Culture of adult rat kidney in saline – embryonic medium with addition of tumour extract. Note expansive character of growth. Portions become detached and grow in various directions over the slide. Forty-eight hours' growth.

FIG. 9.—Culture of adult rat kidney in saline medium + embryonic extract. Eleven days' growth. Note almost entire absence of cell proliferation.

FIG. 10.—Culture of adult rat kidney in saline medium + embryonic extract with addition of autolysed tissue extract. Forty-eight hours' growth. Note extremely rapid proliferation of cells.

FIG. 11.—Culture of adult rat kidney in saline – embryonic medium with addition of extract from tumour 63 extracted in cold without autolysis. Forty-eight hours' growth. Note rapid growth similar to that obtained with the use of an autolysed normal tissue extract.

FIG. 12.—High-power view of portion of culture shown in fig. 11, showing perfectly normal kidney cells with mitochondria.

FIG. 13.—High-power view of portion of last culture, showing perfectly normal mitosis.

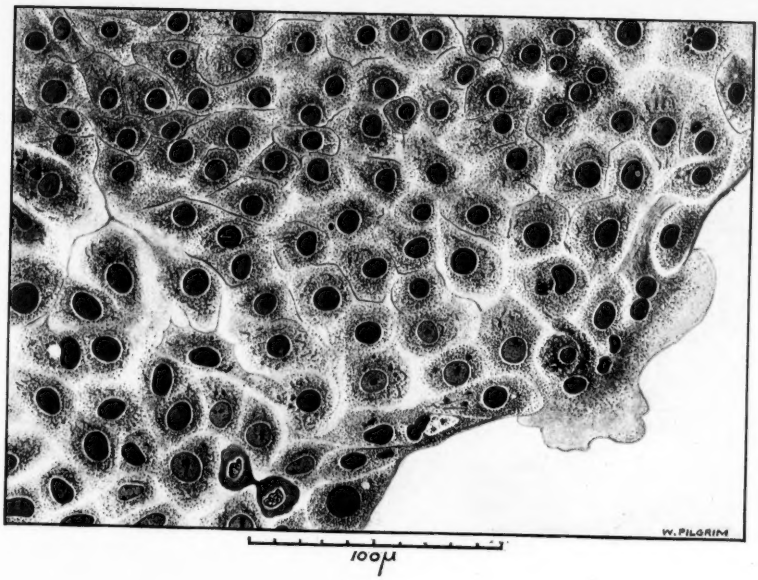

FIG. 1.

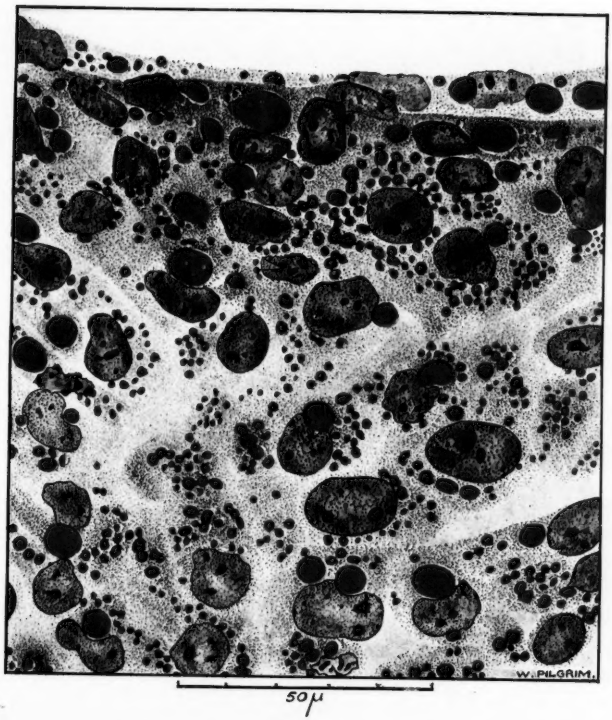

FIG. 2.

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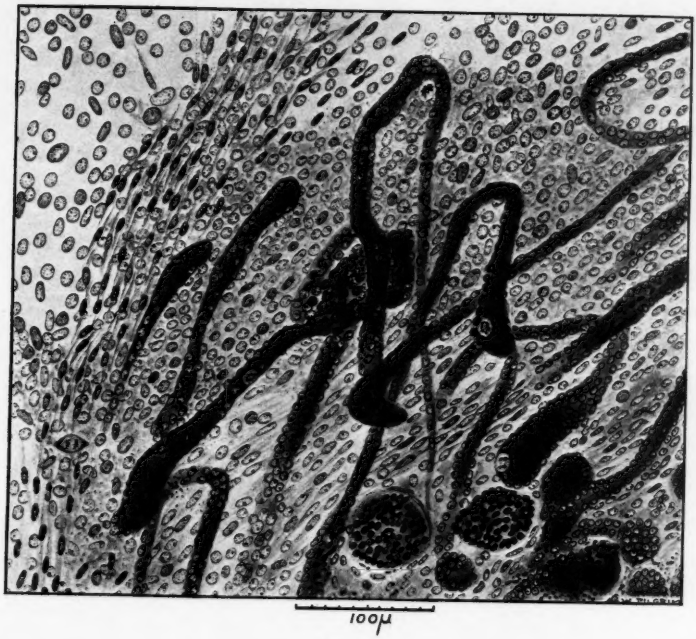

FIG. 3.

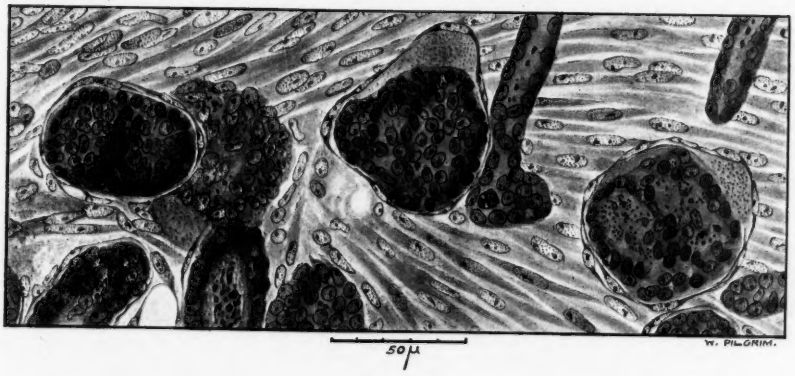

FIG. 3*a*.

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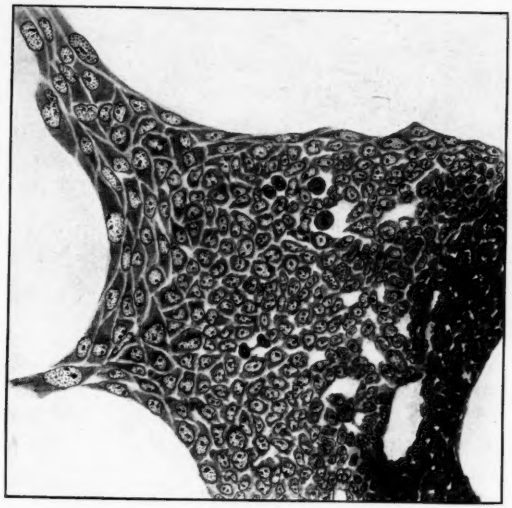

FIG. 4*a*.

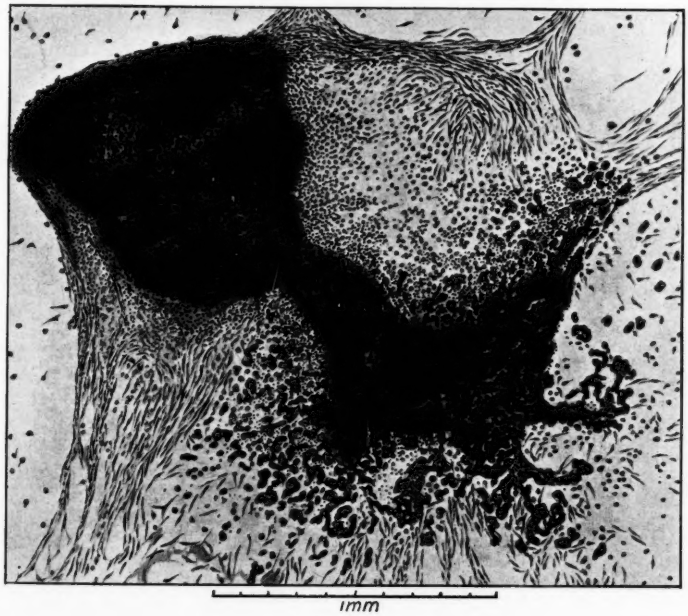

FIG. 6.

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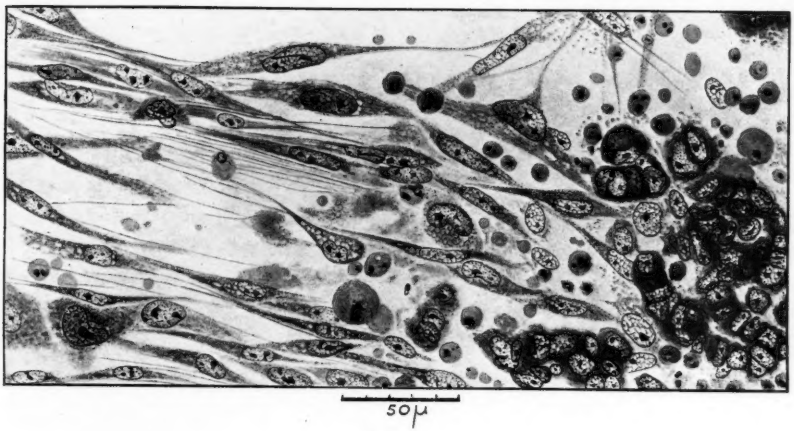

FIG. 7.

FIG. 8.

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FIG. 9.

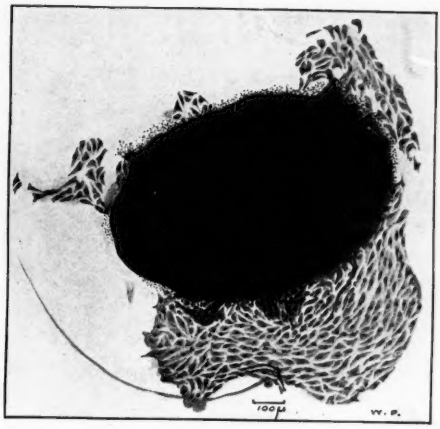

FIG. 11.

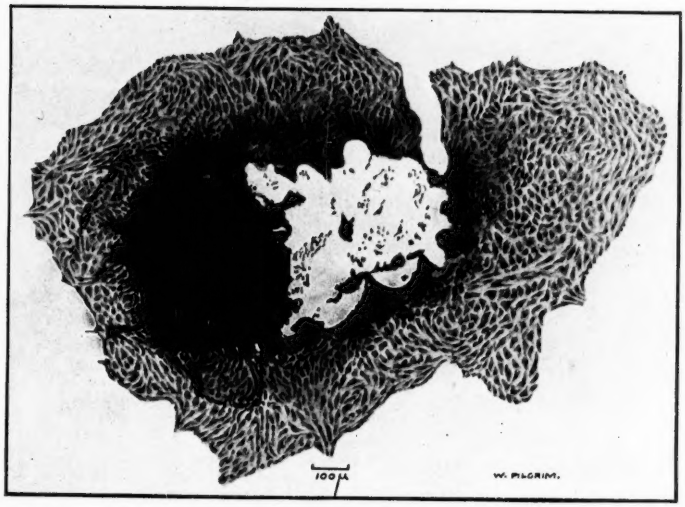

FIG. 10.

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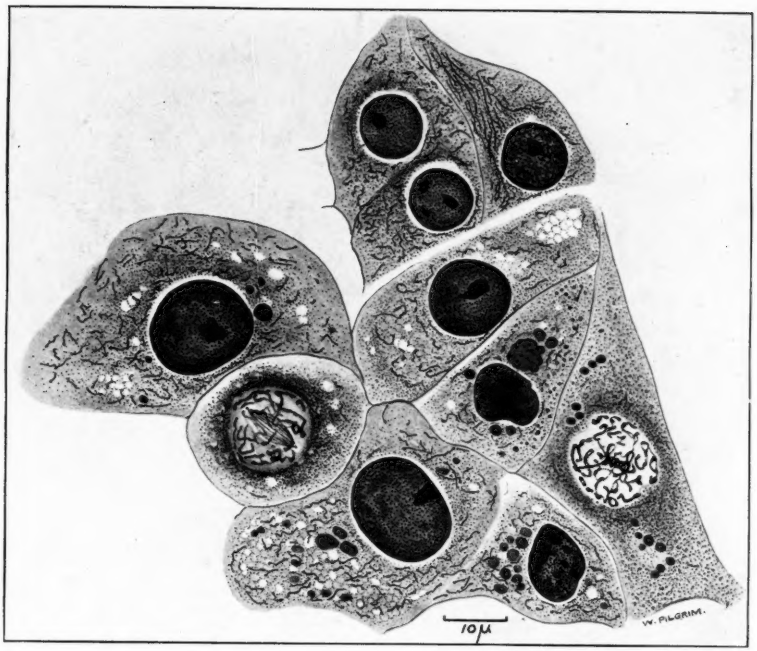

FIG. 12.

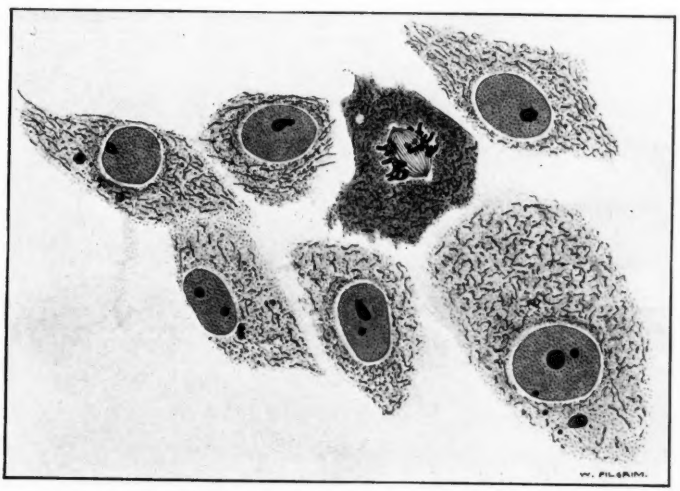

FIG. 13.

Drew.

ON THE MODE OF PRODUCTION OF BACTERIOPHAGE.

ELIZABETH H. LEPPER, M.B., B.S.,
Beit Memorial Research Fellow.
From the Bacteriological Department, Lister Institute, London.

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THE following experiments were undertaken with a view to discovering the conditions necessary for securing a bacteriophage for *B. coli* in its most potent form.

In an investigation into the rate of action of bacteriophage (Lepper, 1923) it had been found that the effect of the lytic substance is very rapid, a large proportion of the bacilli treated being killed in five minutes.

It was also found that guinea-pigs which had been injected intraperitoneally with a mixture of bacteriophage and a lethal dose of *B. coli* lived for several days, whereas the control guinea-pigs which had received the same dose of organisms without the lysin died in a few hours.

It seemed possible, therefore, that if a method could be found for producing bacteriophage for different strains of *B. coli*, beneficial results might be obtained by local treatment. In chronic pyelitis, for instance, an autogenous bacteriophage introduced into the pelvis of the kidney might be of therapeutic value.

Origin of the bacteriophage employed.—The bacteriophage which provided the material for investigation in these experiments was obtained from the urine of a woman suffering from bilateral pyelitis caused by *B. coli communis*.

Specimens of urine were passed through a Berkefeld filter, and varying amounts of the filtrate incubated in peptone water with the autogenous culture obtained from the same urine.

The third sample filtered yielded a positive result, as subcultures on agar made from the mixture after three hours' incubation showed a few large "nibbled" colonies only, growth inhibition being almost complete. After twenty-four hours' incubation subcultures from the mixture showed a good growth of the organism, but many typical "colonies of bacteriophage" or circular patches free from growth.

The strength of this bacteriophage has been considerably increased by passage with the autogenous organism.

The cultural characteristics of the B. coli *concerned.*—When first isolated this organism appeared to be a perfectly ordinary *B. coli*, but in the course of various experiments in which a peptone-water culture, to which no lysin had been added, was always put up as a control to the experimental tubes, it was found that every now and then subcultures from this control tube on agar showed a growth consisting solely of "nibbled" colonies.

This peculiarity of the culture was met with when plating experiments to test the strength of different bacteriophages were being made. On two occasions the control culture, to which no lysin had been added, after growing normally for some hours so that the peptone became definitely cloudy, was

noticed at a later examination to be less cloudy, and was found to contain fewer bacilli per drop than it had done some hours previously.

The culture in fact behaved as a mixture of bacilli and bacteriophage such as d'Hérelle has described as occurring particularly in strains isolated from urine.

The culture when plated yields two types of colony, viz.:

(A) *Round type.*—These are the most numerous, are slightly opaque and raised.

(B) *Crescentic type.*—These are larger, rather transparent flat colonies with irregular edge.

The appearances obtained when the culture is treated with the bacteriophage.—Subcultures from a mixture of the bacillus and the bacteriophage will, in addition to round and crescentic colonies, show large raised opaque colonies which present one or more circular bites at the periphery. These are called "nibbled" colonies, and they appear when the culture has been treated with a strong filtrate. They have been found to be more resistant to the action of the lysin than the other types.

A fourth variety also appears, viz. the mucoid colony, which turns up as a resistant variety during the action of strong lysins, either by itself, or with the "nibbled" form. It is a raised transparent colony which grows rapidly at room temperature, and runs in a mucoid stream down the surface of the agar slope and forms a semi-fluid deposit at the bottom of the tube.

If subcultured and kept on the bench, the "mucoid" form maintains these characteristics, but in the incubator it reverts quickly to the "round" type.

Less active lysins produce the classical appearance of numerous circles free from growth.

Very weak lysins produce a growth which appears to be a mixture of opaque and transparent colonies, resulting in a very uneven-looking slope. This is called the "mixed" culture.

QUANTITATIVE METHODS FOR ESTIMATING THE STRENGTH OF BACTERIOPHAGE.

When a bacteriophage of moderate strength acts on a sensitive organism there is an immediate effect during which a certain number of the bacilli are killed, the number destroyed depending on the strength of the lysin, and a secondary effect in course of which the lysin is reproduced. The exact relation of the secondary to the primary effect has not been definitely worked out.

Of the quantitative methods which have been most used, that of d'Hérelle depends entirely on the secondary effect, that of Maitland (1922) and the somewhat similar method of Otto, Munter and Winkler (1922) aim at combining the results of both effects, while that of Appelmans (1921) depends on the primary effect, if the readings are made at the end of three hours, and on both effects if the tubes are incubated overnight.

None of these methods were selected for the following reasons:

(1) The existence of the zonal phenomenon, described by Ellis (1922), examples of which are given later in this paper, necessitates that to yield quantitative results, the number of organisms used in d'Hérelle's method should be standardised and the period of incubation fixed.

(2) The methods which are based on the appearances resulting when mixtures of bacteriophage and bacilli are grown on agar, are open to the objection that the personal factor must play a large part in assigning to any particular culture a quantitative value, the appearances being so varied that it is not easy to place them in order of magnitude and bacteriophage action. These methods may suffice for comparative results in the hands of an individual worker, but do not furnish a sufficiently accurate standard for quantitative results by different observers.

(3) Appelmans' dilution method requires to be supplemented by subculture. Certain dilutions of bacteriophage and bacilli may remain relatively clear as compared with the control culture to which no lysin has been added, and yet show numerous colonies if subcultured on agar.

As the object of these experiments was to obtain lysins which would have a marked immediate effect on the bacilli, *a method which would measure the actual lethal action was aimed at.*

Details of the method selected.—The method used was based on that employed at the Lister Institute for comparing the strength of disinfectants.

Dilutions of the bacteriophage to be tested are made in 4·9 c.c. peptone-water. To each of these, 0·1 c.c. of an emulsion of bacilli is added so that the total volume amounts to 5 c.c.

It has been found that a dose of organisms suitable for demonstrating the lethal effect of the lysins may be obtained in the following way:

A peptone-water culture is made overnight. In the morning 0·1 c.c. of this culture is delivered, by means of a standardised dropping pipette, into 4·9 c.c. peptone-water and the mixture incubated for three hours. This three-hours-old culture is diluted 1/10 with saline, and 0·1 c.c. of the resulting suspension added to each dilution of bacteriophage and to a control tube containing 4·9 c.c. of peptone-water only. An interval of one minute is made between the inoculation of each tube in order that sufficient time may be available when the subsequent subcultures are made.

Plates made from the control tube give a count varying between 1800 to 2200 colonies per drop (0·02 c.c.).

The tubes containing the lysins and bacilli are kept at room temperature and subcultured at minute intervals at the end of 4 hours and 24 hours, by delivering 0·01 c.c. from each tube into two tubes of lactose-litmus peptone-water, and 0·1 c.c. are also transferred to an agar slope.

These subcultures are incubated for 48 hours.

If no growth occurs in the lactose tubes then the bacilli must have been reduced to less than one per drop; if the bacteriophage has not been sufficiently active to produce this result the agar slopes are useful in showing evidence of bacteriophage action in a particular dilution.

By putting up tubes containing various amounts of carbolic acid and inoculating them at the same time with the same number of organisms, the activity of the bacteriophage can be compared with that of the chemical disinfectant.

EXPERIMENTS TO SHOW THE INCREASE OF BACTERIOPHAGE BY PASSAGE.

EXPERIMENT I.—*To study the effect on a heterogeneous culture.*

The various samples of bacteriophage used in these experiments were obtained by growing the bacillus isolated from a case of pyelitis in broth with some of the filtered urine from the same patient, specimen "E." The filtrate of this culture was called "Ea." This was used for a second passage, was filtered and called "Eb" and so on.

The action of these filtrates on a sensitive bacillus "Merrill" is shown in

Table I. The number of passage cultures which had taken place are given in the second column of the table. Unfortunately some of the samples had been used up in earlier experiments, so that it is not possible to give the complete series.

It will be seen that it is only after the fourth passage that a 1 in 10 dilution of the bacteriophage was able to reduce the bacilli from 1281 per drop (0·01 c.c.) to less than one per drop. The third sample, however, showed evidence of bacteriophage on the agar slope made from both dilutions at the end of two hours, well-marked circles being present. On counting these it was found that the slope from the 1/10 dilution showed 11 circles, while that from the 1/20 dilution showed 16.

In this experiment some of the dilutions were plated at the end of 2½ hours, the number of bacilli left per 1 c.c. being calculated in each case and displayed in column No. 6. In the early experiments plating was thus employed in order to control the results obtained in the fermentation tubes. The plates confirm the tubes except for sample 1/5000 of "Flask I."

TABLE I.—*To Show that Passage Increases the Activity of Bacteriophage.*

The experiment was carried out at 18° C., with a heterogeneous organism. 128,100 bacilli were present in 1 c.c. at beginning of experiment.

Filtration.	No. of passage.	Dilution.	0·01 c.c. into 2 lactose tubes. 2 hours.	0·01 c.c. into 2 lactose tubes. 24 hours.	Bacilli per 1 c.c. at 2½ hours.
E	None	1/5	+ +	+ +	*
		1/20	+ +	+ +	128,500
Ec	3rd	1/10	+ +	+ +	*
		1/20	+ +	+ +	95,500
Ed	4th	1/10	− −	− −	20
		1/50	+ +	+ +	450
Ee	5th	1/5	− −	− −	10
		1/10	− −	− −	...
		1/20	+ +	− +	200
Round flask	6th	1/10	+ +	− +	20
		1/50	+ +	− +	150
Flask I	9th	1/100	− −	− −	...
		1/500	− +	− +	130
		1/1000	− +	+ +	...
		1/5000	− +	+ +	950
Carbolic acid	—	1/200	− +	− −	...
		1/400	+ +	+ +	...

+ = Acid and gas in one lactose tube. ++ = Acid and gas in both.
* Colonies too numerous.

Summary of Table I.—The table shows that the fourth, fifth and sixth filtrates are stronger than the third, while the ninth is considerably stronger than any of the others.

EXPERIMENT II.—*To study the effect on the autogenous culture.*

Having found a definite increase in strength of the bacteriophage as evidenced by its action on a heterogeneous culture it was of interest to find out—

(*a*) Whether the autogenous culture was more susceptible than the heterogeneous, and (*b*) whether there was any difference in the activity of the bacteriophage towards the two types of bacilli which could be isolated from the autogenous strain.

The results are shown in Table II.

TABLE II.—*To Show the Action of Various Samples of Bacteriophage on the Autogenous Strain.*

Experiment at room temperature.

Filtrate dilution.	No. of passage.	Type of bacillus.	No. of bacilli per drop (0·02 c.c.) at end of 2½ hrs. Experimental.	Control.	0·01 c.c. into lactose— 4 hours.	24 hours.
E 1/10	None	Round	4345	3450	+ +	+ +
		Crescentic	3400	3838	+ +	+ +
Ec 1/10	3rd	Round	798	3450	— —	— +
		Crescentic	3750	3838	+ +	+ +
Ee 1/10	5th	Round	0·2	2170	— —	— —
1/100		„	...	...	— —	— —
Round flask						
1/10	6th	Round	0	2170	— —	— —
1/100		„	...	...	— —	— —
Flask I						
1/500	9th	Round	2	2170	— —	— —
1/1000		„	...	...	—	— —
1/5000		„	...	...	—	— —
Carbolic 1/250	...	Round	...	...	— +	— —
Acid 1/500	...	„	...	...	+ +	+ +

Summary of Table II.—(1) It will be seen that the various samples of bacteriophage do act in higher dilutions on the autogenous strain. Filtrate "Ee" for instance entirely killed the autogenous culture in a dilution of 1/100, whereas a dilution of 1/20 of the same filtrate was not lethal for the heterogeneous strain.

The action of higher dilutions of the strongest bacteriophage, viz. that obtained after the ninth passage, is shown in Table III (see below). Its action on the autogenous strain is about ten times greater than that on the heterogeneous culture.

(2) There is evidence that the round type of colony is more sensitive than the crescentic, the 1/10 dilution of "Ec" killing off three-fourths of the bacilli of the round type in 2½ hours, whereas the bacilli of the crescentic type were not appreciably reduced in that time.

(3) Comparing the effect of the bacteriophage with that produced by the carbolic acid, it will be seen that, during the first 24 hours, the action of the bacteriophage is more powerful than a 1/500 dilution of carbolic acid.

EXPERIMENT III.—*To study the effect of the strongest bacteriophage on the autogenous culture during a longer period of time.*

This sample of bacteriophage was obtained after the ninth passage, and is referred to as "I."

Dilutions of this sample were made from 1/50 to 1/2,500,000 and put up with the autogenous culture at room temperature. Subcultures were made at the end of 22 and 47 hours. The tubes were then placed in the incubator for three hours to see if any resistant bacilli would grow up in the tubes which had so far proved sterile. Subcultures on agar were then made.

The results are shown in Table III.

TABLE III.—*To Show the Action of Bacteriophage "I" on the Autogenous Strain.*

Number of bacilli per 1 c.c., 115,250. Experiment performed at 20·5° C. for 46 hours, and finally at 37° C. for 3 hours.

Dilution of bacteriophage.	Subculture after 22 hours at room temperature.		Subculture after 46 hours at room temperature and 3 hours at 37° C.	
	Lactose.	Agar.	Lactose.	Agar.
1/50	− −	No growth	− −	No growth.
1/500	− −	„	− −	„
1/2500	− −	„	− −	„
1/5000	− +	1 "nibbled" colony.	+ +	1 "nibbled" colony.
1/25,000	− −	1 „	+ +	±
1/50,000	− +	±	+ +	+
1/250,000	+ +	+ +	...	+ + +
1/500,000	− +	±	+ +	2 "nibbled" colonies.
1/2,500,000	+ +	1 large colony	...	2 „

Note.—In lactose tube + = acid and gas. In agar tube + + + = normal growth, + + = 2/3 normal growth, + = 1/3 normal growth, ± = 1/6 normal growth.

Summary of Table III. — (1) The dilutions 1/50, 1/500 and 1/2500 remained sterile. (2) It will be noted that from the appearances on agar the dilutions 1/2,500,000 and 1/500,000 appear to have a stronger action than the 1/250,000 dilution. This phenomenon had not been observed when dealing with the heterogeneous strain. The cause of this zonal effect will be discussed later, but it gives rise to a very real difficulty in comparing the strengths of samples of bacteriophage on the autogenous strain.

EXPERIMENTS DEALING WITH THE PRODUCTION OF LYSIN IN THE COLD.

It had been found in some experiments on the rate of action of bacteriophage that the sample "I," which has been used as the stock in all the following work, would act quite efficiently at 0°C., reducing the number of bacilli from 2935 per drop (0·02 c.c.) to 2 per drop in an hour's time. As no growth occurs in the control culture at this temperature it should be possible to find out whether the destruction of a large number of bacilli was in itself sufficient to increase the amount of lysis present.

EXPERIMENT I.—*To find out whether increase in the activity of a bacteriophage takes place when the sensitive organisms with which it has been in contact have been killed.*

Three flasks each containing 100 c.c. of broth were taken; one was kept as a control, and 2 c.c. of bacteriophage "I" added to each of the other two—a dilution of 1/50.

Two agar slopes of the autogenous culture which had been incubated for three hours were emulsified in 5 c.c. saline; 1·6 c.c. of this emulsion were added to each flask, rendering them distinctly cloudy.

One of the flasks containing lysin was put in the incubator, the other two were put in the cold room at 0°C.

At the end of two hours the incubated flask was distinctly less cloudy than the control in the cold room, while the experimental flask which had been in the cold room showed no clearing.

Next day the incubated flask had become very cloudy while the flasks in the cold room had not changed in appearance.

At the end of 21 and 41 hours 20 c.c. of each of the experimental flasks was filtered, and on the sixth day 20 c.c. from the incubated flask.

The activity of these filtrates was tested on the heterogeneous strain and the results are shown in Table IV. When comparing the action of the bacteriophage in these filtrates with that of the stock "I" from which they were derived, allowance must be made for the dilution of 1/50 which took place when 2 c.c. of the stock lysin was added to the 100 c.c. of broth.

TABLE IV.—*To Show the Action of the Filtrates Obtained in Experiments I and II.*

Tested on "Merrill" at room temperature, subcultures being made into lactose at end of 24 hours. Control bacteriophage "I" 1/100 − −; 1/1000 − −; 1/2000 − +.

Source of filtrate.	Final dilution compared with "I."	Day on which filtration took place. 1st.	2nd.	4th. Cold.	4th. Incubated.	6th.
Flask kept in incubator .	1/500 .	− −	− +	...	...	+ +
	1/5000 .	− −	+ +	...	...	+ +
	1/50,000 .	+ +	...	...	...	...
Flask kept in cold room .	1/500 .	+ +	+ +	+ +	− −	...
	1/2500 .	+ +	...	+ +	− −	...
	1/10,000 .	...	...	...	+ +	...

Summary of Table IV.—(*a*) Incubated flask: (1) It will be seen that there is a slight absolute increase in the bacteriophage-content at the end of 21 hours.

(2) At the end of 41 hours there was an absolute decrease.

(3) At the end of 6 days there was not enough lysin to be detected by this method.

(*b*) Flask kept in the cold room: (1) The bacteriophage disappeared rapidly, and at the end of 21 hours there was not enough to be detected by this method.

EXPERIMENT II.—*To find out the effect of incubation on a mixture of bacteriophage and bacilli which had been in contact at 0°C. for four days.*

On the fourth day, as no clearing had occurred in the lysin-containing flask in the cold room, the following experiment was made.

Two test-tubes of approximately equal size were selected and 20 c.c. of the

contents of the experimental flask from the cold room put into each; a few drops were also placed on an agar slope.

One tube was put in the incubator while the other was returned to the cold room.

At the end of one hour there was distinct clearing in the incubated tube, and at the end of three hours it was practically clear; both samples were then filtered.

The agar slope made before incubation proved sterile, and it is of interest that although the organisms had been killed, no clearing of the culture occurred until incubation had taken place.

These filtrates were put up with strain "Merrill" and the results shown in Table IV.

Summary of the results obtained in Experiment II.—(1) The incubation of a mixture of bacteriophage and bacilli which had been kept in the cold and in which the strength of the lysin had become much reduced led to the reappearance of the bacteriophage in its original strength.

(2) No actual increase could be proved to occur, although a large number of bacilli had been killed and lysed.

EXPERIMENT III.—*To find out whether a large number of bacilli killed and lysed would give more evidence of increase in bacteriophage than a small number.*

It was thought possible that a sufficiently large number of bacilli had not been used in the previous experiment, which was therefore repeated in the following way:

An emulsion was made in 7 c.c. of saline of the growth from 7 agar slopes. Two flasks, each containing 100 c.c. of bacteriophage "I" diluted 1/50 in broth, were prepared; 0·5 c.c. of the bacillary emulsion was added to one flask, "W," and 5 c.c. to the other, "S." Both flasks were kept in the cold room for 10 days when samples from each were filtered, (1) when taken direct from the cold room, and (2) after three hours' incubation. Marked clearing took place in both the incubated samples but neither became entirely clear.

Plates made from the experimental flask which had received 5 c.c. of emulsion, and from a control flask to which no lysin had been added, showed that at least 200,000 bacilli per drop (0·02 c.c.) had been destroyed by the bacteriophage.

The filtrates were tested on the heterogeneous and on the autogenous strains and confirmed the results obtained in the previous experiments.

Summary of results of Experiment III: (a) *Filtrates made direct from the cold room.*—(1) The amount of bacteriophage in both flasks was reduced.

(2) The reduction was more marked in "S," the flask which had received the larger number of organisms.

(b) *Filtrates which had been incubated for three hours.*—(1) The activity of the bacteriophage had been restored to its original strength for the autogenous culture.

(2) With the heterogeneous culture the original activity was not entirely

regained, but there was an increase in activity as compared with the action of the filtrates made direct from the cold room. This was particularly the case with the incubated sample from flask "W" which had received the smaller number of organisms.

EXPERIMENT IV.—*To find out whether a higher temperature would favour the reappearance of the lytic substance.*

When flask "S" had been in the cold room for fourteen days two samples of 10 c.c. were taken and placed in two test-tubes of equal size. One tube was placed in a water-bath at 47° C. for two hours, while the other was kept in a bath at 58° C. for the same time. 5 c.c. of each sample was passed through a Berkefeld filter. These filtrates were then tested on strain "Merrill."

Result of Experiment IV.—The amount of lysin found in filtrates which had been heated to 47° C. and 58° C. for two hours was less than that found in filtrates which had been incubated for three hours.

EXPERIMENT V.—*To find out whether the filtration through a porcelain filter had removed the bacteriophage in the previous experiments.*

The remaining half of each sample of "S" which had been heated to 47° C. and 58° C. in Experiment IV was subcultured and found to be sterile. They were tested on "Merrill."

Result of Experiment V.—These samples did not show any greater activity than the filtered specimens from the same source.

Conclusions drawn from these experiments.—(1) Bacteriophage kept in the cold in contact with bacilli becomes inactive.

(2) The larger the number of bacilli present, the more completely is the bacteriophage action lost.

(3) The bacilli which have been treated in the cold with bacteriophage, even if some weeks old, undergo lysis subsequently on incubation, and the active substance reappears in the broth. This lysin is undiminished in strength for the autogenous bacillus.

(4) Temperatures higher than 46° C. do not favour the reappearance of lysin.

(5) There is no evidence that the death and subsequent lysis of a large number of bacilli can lead to an increase in the amount of bacteriophage present.

EXPERIMENTS TO INVESTIGATE THE DISAPPEARANCE OF BACTERIOPHAGE WHICH TAKES PLACE IN THE COLD WHEN IN CONTACT WITH SENSITIVE BACILLI.

EXPERIMENT I.—*To find out whether the bacilli have combined with the bacteriophage.*

Four centrifuge tubes were filled to a mark with broth from flask "S," now 15 days old, and a test-tube-full to act as control was kept at room temperature.

The flask was plated and found to contain 30,050 bacilli per drop (0·02 c.c.).

The tubes were centrifuged, the supernatant fluid pipetted off into a test-tube and the deposit washed three times with peptone-water. The washings were saved.

Two of the centrifuge tubes were now filled to the mark with sterile broth, the other two with the original supernatant fluid.

There were thus three preparations—

(1) The unaltered sample kept at room temperature denoted "R."

(2) The washed bacilli in fresh broth denoted "B."

(3) The washed bacilli in supernatant fluid denoted "S."

All three specimens were cloudy. They were placed in the incubator and at the end of three hours were almost clear.

After twenty hours' incubation all the tubes had become cloudy, and subcultures made from them on agar showed a mixture of large "nibbled" colonies and small transparent colonies.

The tubes were now filtered.

If it had been possible, by washing, to remove the bacteriophage which had been in contact with the bacilli, then, of these specimens, "B" ought to show no evidence of lytic action, "S" very little, viz. that due to the very small amount of bacteriophage left in the supernatant fluid which had not entered into combination with the organisms, and "R," the control, should show much the strongest bacteriophage effect.

The filtrates were tested on "Merrill" and the results are shown in Table V.

TABLE V.—*To Show the Lytic Action of the Filtrates obtained in Experiment I, tested on "Merrill" at Room Temperature: Subcultures made at the end of 24 hours.*

Control bacteriophage "I" 1/500 lethal, 1/2500 not lethal.

Source of sample.	Final dilution compared with "I."	Subcultures on lactose. "R" untreated.	"B" washed + broth.	"S" washed + supernatant.
Flask S, containing 1/50 "I" and 5 c.c. bacillary emulsion, cold room 15 days	1/500	− −	− −	− −
	1/2500	− −	− +	− −
	1/5000	+ +	+ +	− −

Summary of Table V.—(1) It was found that all three samples showed evidence of the presence of bacteriophage.

(2) Not only was a bacteriophage present, but all the filtrates showed a lysin which was more active than the control.

(3) This increase was most marked in the case of "S," which contained the bacilli which had been washed and put up with the supernatant fluid.

(4) The filtrate from the washed bacilli in fresh broth gave almost as strong a lysin as the control untreated organisms. It had not, therefore, been possible by washing to alter the relationship existing between the bacteriophage and the organisms.

(5) Why the washed bacilli which had been incubated with the original supernatant fluid should have produced the strongest lysin is not clear, but a possible explanation may lie in the number of bacilli which were present in

each specimen when they were placed in the incubator. Details regarding the plates which were made from these specimens are as follows:

Flask "S," which had been in the cold room for 15 days, contained 30,050 bacilli per drop (0·02 c.c.).

Time and treatment.	Number of bacilli per drop. Sample "R."	Sample "B."	Sample "S."
Three hours at room temperature.	1000	45	1450
After 3 hours' incubation . .	2·5	1·6	2
„ 24 „ „ . .	9,440,000	19,800,000	7,500,000

It will be seen that in each case a very large number of bacilli, presumably those least resistant, had been killed off at room temperature. A further decrease in the number present occurred during the first three hours of incubation. The subsequent development of the resistant type of bacillus led to a definite increase, as compared with the control, of the amount of lysin present.

Conclusions from Experiment I.—(1) There is evidence that, in the cold, bacteriophage enters into combination with sensitive bacilli.

(2) The lysin so combined cannot be entirely removed by repeated washing.

(3) An increase in the amount of lysin was associated with the proliferation of a very resistant type of bacillus.

EXPERIMENT II.—*To repeat the previous experiment at a lower temperature and without incubation of the bacilli.*

When flask "S" had been in the cold room for seven weeks a sample was centrifuged and washed three times with broth which had been cooled to 0° C., so that the temperature of the organisms might be kept as low as possible and lysis of the bacilli reduced to a minimum.

The deposit was sucked dry after each washing, so that some organisms were unavoidably carried over from the deposit. These fluids were kept.

After the third washing the deposit of bacilli was shaken up with broth. This, the original supernatant fluid, and the washings were cultured on agar. The tubes were then placed in the cold room.

All the cultures showed a growth of slightly "nibbled" colonies.

After 24 hours in the cold the tubes were heated at 58°C. for 1½ hours and found to be sterile.

These five preparations, the supernatant fluid, the three washings and the deposit of bacilli in broth, were tested on the autogenous culture.

Summary of the results obtained in Experiment II.—(1) Subcultures on agar from mixtures of these filtrates with the autogenous culture which had been kept in the cold room for 24 hours showed no evidence of bacteriophage.

(2) Subcultures on agar made after three hours' incubation of the mixtures all showed evidence of bacteriophage.

(3) After 28 hours' incubation of the mixtures this bacteriophage effect was increased, the most marked result being obtained in the tube containing the washed deposit of bacilli.

(4) The third washing, although it was a dilution of at least 1/6,000,000 of

the original fluid in the flask, showed much the same appearances as those given by the supernatant fluid.

Conclusions from Experiment II.—(1) This experiment confirms the previous one that in the cold bacteriophage combines with sensitive bacilli.

(2) Repeated washings do not entirely remove the lysin from the organism.

(3) It shows that the lysin carried over with the bacilli is able to produce a bacteriophage effect on a fresh inoculation of organisms.

(4) There was a very marked difference in the amount of lysin which could be demonstrated in this experiment and that found in the preparations in the first experiment, in which the resistant bacilli had been incubated, and had increased in number.

EXPERIMENTS DEALING WITH THE PRODUCTION OF LYSIN AT 37°C.

As all efforts to produce increase of bacteriophage without growth of the baciili had failed, observations were made on the production of lysin which resulted when varying proportions of bacteriophage and bacilli were incubated together and samples filtered at intervals.

EXPERIMENT I.—*To investigate the production of lysin resulting from the action of varying strengths of bacteriophage on different numbers of organisms.*

Three flasks each containing 200 c.c. of broth were taken. To two of these, "A" and "B," 1 c.c. of bacteriophage "I" was added—a dilution of 1/100; to the third, "C," 1 c.c. of a 1/10 dilution, so that it contained 1/1000 lysin.

Two agar slopes of the autogenous culture which had been incubated for three hours were emulsified in 5 c.c. saline and 1·6 c.c. added to flasks "A" and "C," only 0·16 c.c. being added to "B."

The flasks were placed in the incubator and 20 c.c. of each filtered at the end of 3, 24 and 72 hours.

These filtrates were tested against "Merrill" (see Table VI).

TABLE VI.—*To Show the Action of the Filtrates obtained in Experiment I on the Heterogeneous Strain "Merrill," Tested at Room Temperature; Tubes Subcultured into Lactose at the end of 24 hours.*

The control bacteriophage "I" 1/500 − −, 1/2500 + +.

Source of filtrate.	Final dilution compared with "I."	Number of hours sample was incubated before filtration. 3 hours.	24 hours.	72 hours.
Flask "A" containing bacteriophage 1/100; bacillary emulsion 1·6 c.c.	1/500	− −	− −	− +
	1/5000	− −	− −	− +
	1/25,000	+ +	+ +	+ +
Flask "B" containing bacteriophage 1/100; bacillary emulsion 0·16 c.c.	1/500	− −	− −	− −
	1/5000	+ +	− −	− −
	1/25,000	...	+ +	+ +
Flask "C" containing bacteriophage 1/1000; bacillary emulsion 1·6 c.c.	1/5000	− −	+ +	...
	1/10,000	− −	...	...
	1/50,000	+ +	+ +	...

Summary of Table VI.—(1) An increase in the strength of the lysin was detectable in each case.

(2) When the amount of lysin added was 1/100, as in "A" and "B," the bacteriophage in the flask containing the larger number of bacilli "A" reached a maximum sooner and disappeared more rapidly.

(3) When the amount of lysin was 1/1000, as in flask, "C," and the number of bacilli the same as "A," the increase in lysin was distinct at the end of three hours, but at the end of 24 hours was hardly greater than the control.

A subculture on agar made from this flask at the end of three hours' incubation showed a few "nibbled" colonies, but no enumeration was made.

Doerr and Gruniger (1922), using a high dilution of lysin and a small number of organisms and estimating the strength of the lysin and the number of bacilli present in their mixture at frequent intervals, found that the bacilli proliferated normally for the first four hours and then rapidly decreased in number; the lysin remained unaltered for the first $2\frac{1}{2}$ to 3 hours, and then rapidly increased in titre, reaching a maximum in 4 hours. The lysin increased 100,000-fold in two hours (titrated by Applemans' (1921) method).

I have calculated that I should have to use the bacteriophage "I" in a dilution of 1×10^{-7}, or 10^{-8} for it to approximate in value to the lysin used by Doerr and Gruniger. I hope to be able to carry out further experiments with such dilutions at a later date.

Conclusions from Experiment I.—Much more work requires to be done to determine the exact significance of these results, but they suggest that bacteriophage is reproduced either by a certain variety of the organisms or at a certain stage in their growth.

They show that favourable conditions may be produced by varying the amount of lysin, the number of bacilli, or the time at which filtration is carried out.

EXPERIMENT II.—*To see whether the resistant type of bacillus was suitable for the production of bacteriophage in a passage experiment.*

Subcultures were made from a culture of "nibbled" colonies which had been obtained from flask "A" at the end of 24 hours' growth, and an experiment exactly similar to that described undertaken, the flasks in this case being "AR," "BR" and "CR."

These filtrates were tested against both the heterogeneous and the autogenous strains.

Summary of results obtained from Experiment II.—(1) With the heterogeneous strain only the flask "CR" containing the 1/1000 lysin showed evidence of an increase in the amount of bacteriophage present.

(2) When tested on the autogenous strain all the filtrates showed increased activity of lysin.

(3) The effect of these lysins was observed over a period of 17 days. Several examples of late sterilisation of the culture occurred with high dilutions of lysin, confirming the previous observation that certain proportions of lysin and bacilli are more favourable for the production of bacteriophage than others. Ellis called attention to this zonal phenomenon as occurring when visible lysis

is taken as the criterion of bacteriophage effect. In my experiments it was particularly noticeable with lysins that were not strong enough to prevent the growth of the resistant type of bacillus, and especially when the tubes were kept at room temperature, so that changes in the number of organisms present took place slowly.

An example of the zonal effect is given in Table VII.

TABLE VII.—*To Show the Effect of the Filtrate Obtained from Flask "AB," Containing the Resistant Type of Bacillus and 1/100 Bacteriophage, Filtered after Three Hours' Incubation and Tested on the Autogenous Strain at Room Temperature.*

Final dilution compared with bacteriophage "I."	Subcultured on lactose after interval of —			Agar 17 days.
	1 day.	4 days.	17 days.	
1/5000	− −	− −	− −	No growth.
1/50,000	− +	− −	− −	"
1/250,000	− +	− −	+ +	Good growth.
1/500,000	− +	+ +	− +	No growth.

EXPERIMENT III.—*To find out whether the resistant type of bacillus without the addition of lysin could give rise to bacteriophage.*

A saline emulsion of some "nibbled" colonies obtained on subculture from flask "A" was made, and 0·7 c.c. put in a flask containing 100 c.c. of broth. No lysin was added. The flask was incubated.

Twenty c.c. of this culture was filtered at the end of 3 and 5 hours and on the second and fourth day.

These filtrates were put up against the autogenous culture (Table VIII). One series of tubes was kept at room temperature for 24 hours and then subcultured, the other series in the incubator for three hours.

TABLE VIII.—*To Show the Action of Filtrates Made from a Broth Culture of the Resistant Type of Bacillus on the Autogenous Strain.*

Length of incubation before being filtered and dilution.	Appearances of subcultures on agar.	
	Mixtures kept 13° C. 24 hours.	Incubator 3 hours.
3 hours 1/5	Very small "nibbled" colonies	12 "nibbled."
1/50	Good growth, many circles of "bacteriophage"	...
5 hours 1/5	A few small colonies	6 "nibbled."
1/50	Good growth, many circles	...
48 hours 1/5	A few very small colonies	No growth.
1/50	Good growth, many circles	...
96 hours 1/5	Good growth, many circles	3 small colonies.
1/50	Good growth, a few circles	...

Summary of Table VIII.—(1) All the subcultures on agar from both series showed evidence of the presence of bacteriophage.

(2) The sample which had been filtered on the second day was the most active.

Conclusions from Experiment III.—Without further experiments it is impossible to be certain whether this bacteriophage has been produced by the resistant bacilli, or has been carried over by them from the strong bacteriophage in flask "A" with which they have been in contact. Flask "A" was lethal in a dilution of 1/2500.

As there was evidence of an increasing amount of lysin up to the end of 48 hours, it is possible that bacteriophage was really produced by these organisms.

As, however, the resistant type of bacillus, on subculture, always tends to revert back to the non-resistant type, it is extremely difficult to devise an experiment to prove this point.

GENERAL CONCLUSIONS.

(1) Experiments to produce an increase in the strength of a given bacteriophage by allowing it to kill and subsequently lyse sensitive bacilli have proved negative.

(2) There is some evidence that increase in lysin is associated with proliferation of the resistant type of bacillus.

(3) There is evidence that the proportions existing between the strength of lysin used and the number of bacilli present, and the length of time that the lysins act, are all of importance in the production of bacteriophage.

(4) These observations explain the zonal phenomenon which is seen when varying dilutions of a bacteriophage are put up with the autogenous culture.

(5) Bacilli acted on in the cold by a bacteriophage enter into some combination with it. This has been shown to occur with dead organisms by Jaumain and Meulemain (1922). The bacteriophage is again set free on lysis of the bacilli and is increased in amount if the organisms are then allowed to proliferate.

(6) These observations explain the so-called "mixed" cultures which have been isolated from urine and their peculiar variations in growth.

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THE DETECTION OF *SPIRONEMA PALLIDUM*.

J. E. BARNARD.

From the Department of Applied Optics, National Institute for Medical Research, London.

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AMONG the methods of detecting *Spironema pallidum* there is one that may be regarded as quantitative, and which, therefore, does offer reasonable certainty in diagnosis. This is that recommended by L. W. Harrison ('A Manual of Venereal Diseases,' p. 95), in which the relation of the number of spirals to a given length is determined. The standard suggested is the diameter of a red blood-cell, and seven complete coils of the spironema should be counted in this length. Any number more or less than this renders it improbable that *S. pallidum* is being observed, and when taken in conjunction with other known characteristics it may be regarded as definitely confirmatory. The difficulty of applying this method under ordinary circumstances is obvious, as either there may be few blood-cells in the material, or a long search may not result in seeing a cell and a spironeme in convenient proximity. With a view to overcoming this objection to an otherwise valuable aid the method to be described has been evolved, and has been found easy in manipulation and constant in results. The only appliance necessary, in addition to the ordinary microscopical equipment for dark-ground illumination, is a graticule, which is placed on the diaphragm in the ocular in the same way that an eye-piece micrometer is disposed. It is necessary to make sure that the position of the diaphragm is such that the pattern of the graticule is sharply defined when the ocular is in use. This is not invariably the case, and it may be necessary for some slight adjustment to be carried out by an optician to obtain the desired result. The pattern and size of the graticule is seen in Fig. 1. It consists of a photo-

FIG. 1.

graphic image with a semitransparent ground on which a number of clear spaces are regularly disposed, so that a large proportion of these spaces are always in the field of view. The purpose is to so arrange matters that each clear circle occupies the same area in the field that would be occupied by a red blood-cell. It is obvious that this is dependent on the focal length of the objective in use, and to get a satisfactory result a graticule should be supplied of suitable pitch for each objective if more than one is generally used. The one figured is suitable for a Leitz $\frac{1}{12}$ in. oil-immersion suitably stopped down. In any case some slight adjustment is necessary before the method is ready for use. A normal red blood-cell should be observed, and if its image exactly fills one clear circle then adjustment is correct. Should it not do so the draw-tube of the microscope must be moved either up or down as required until the desired result is obtained. The necessary adjustment should not exceed five millimetres in either direction. Should more movement than this be required the graticule is not of the right pitch and should be exchanged by the makers for another. The correct tube-length with a particular objective and ocular having once been determined, it is only necessary to set it at the same position each time a search is to be made. When all is in correct adjustment the image appears as in Fig. 2. It will be seen that the spirals to be counted are brighter than those outside the circle and this greatly facilitates the counting. After some little experience it is possible to tell without actual counting whether the required seven coils are within the circle, or, what is equally important, whether more or less than this number are there. The method may be applied to other spirochætes where the spacing of the coils is characteristic. It is sometimes difficult with an indifferent mechanical stage to move the object exactly to the required position, but this can often be overcome by rotating the eyepiece when a circle is likely to cross the object.

FIG. 2.

One possible source of error arises when a light of too great intensity is used. In this case irradiation from the blood-cell takes place, and it appears larger than it should do. In such a case the light must be reduced until the cell is only bright enough to be seen comfortably. This may be effected by interposing one or more pieces of neutral or coloured glass between the microscope and the illuminant.

Suitable graticules can be obtained from Messrs. Hawksley & Sons, Wigmore Street, W.

INVESTIGATION OF THE ÆTIOLOGY OF TYPHUS FEVER, ESPECIALLY UNDERTAKEN FOR THE EGYPTIAN GOVERNMENT IN THE PUBLIC HEALTH LABORATORIES, CAIRO.

J. A. ARKWRIGHT, M.D., AND THE LATE A. W. BACOT, F.E.S.

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THE work of which the following paper is an account was carried out during the early months of 1922 in the Laboratories of the Public Health Department at Cairo. It was unfortunately never completed on account of the illness of both the workers from typhus fever. The observations and experiments made will be best considered under three headings: those made—

(1) On the blood of men and guinea-pigs infected with typhus fever.

(2) On the infection of lice with typhus virus and rickettsia.

(3) On lice collected from apparently healthy persons in Cairo.

The virus used for experimental purposes was obtained in the form of blood from two human cases of typhus fever.

OBSERVATIONS ON TYPHUS VIRUS IN BLOOD.

An attempt was made to determine whether the virus was especially closely associated with the blood-platelets. With this object the following experimental inoculations were made into guinea-pigs. All the inoculations of blood and platelets were made intraperitoneally. The sources of the typhus virus used and the experiments carried out were as follows:

Case 3.—M. S—, male, 24 years, admitted February 13th, which was supposed to be the 13th day of the disease, very ill with typhus fever; died February 14th. Weil-Felix reaction positive.

February 13th, 10 c.c. of citrated blood (9 c.c. of blood and 1 c.c. of 5 per cent. citrate of sodium) was received at the laboratory. 6 c.c. of the blood was centrifuged for 10 minutes, 3 c.c. of colourless turbid plasma was pipetted off and again centrifuged for 30 minutes till clear. The deposit examined microscopically in a thick drop after drying and staining consisted almost entirely of platelets; a few red blood-corpuscles and very few lymphocyte nuclei were seen. The deposit of platelets was emulsified in salt solution and made up to 3 c.c.

The following inoculations were made:

Guinea-pig 3 received on 13 . ii . 22, 3 c.c. of whole blood. Result negative, and a subsequent immunity test made on 5 . iv caused a typical febrile attack.

Guinea-pig 4 received on 13 . ii . 22, 2 c.c. of platelet emulsion. Result positive; on 24 . ii (11th day) a febrile reaction began and reached 40° C. Passage to Guinea-pigs 11 and 12 gave positive results and the virus was also carried on through 9 guinea-pigs in series.

Guinea-pig 5 received on 13 . ii . 22, 0·5 c.c. of platelet emulsion; result

positive. On 24 . ii a febrile reaction occurred rising to 40° C. Passage was made with platelet emulsion from Guinea-pig 5 to Guinea-pig 9, which reacted well, but when tested for immunity gave a late reaction, causing some doubt as to the completeness of the immunity.

Guinea-pig 6 received on 13 . ii . 22, 0·1 c.c. of platelet emulsion. Result negative. No marked febrile reaction nor immunity resulted.

Case 4.—Admitted 1 . iii . 22 in the second week of illness; temperature 39° C. Weil-Felix reaction positive; recovery.

Experiments with blood and platelets of Case 4 on 2 . iii . 22 similar to those with the blood of Case 3 gave uncertain results, except that 1 c.c. of whole blood produced a good reaction in Guinea-pig 13, and from this animal the disease was successfully passed on in guinea-pigs in series to the seventh inoculation from man. The other inoculations, viz. 3·5 c.c. of whole blood and 0·1, 0·5 and 2·0 c.c. of platelet emulsion, were followed by results which were uncertain from various causes.

Guinea-pigs.—Inoculations were made with platelets from four typhus guinea-pigs: Guinea-pig 9 inoculated with 1·0 c.c. of platelets from Guinea-pig 5; result positive, immunity test doubtful. Guinea-pig 10 inoculated with 0·1 c.c. of platelets from Guinea-pig 5; result negative, no immunity. Guinea-pig 12 inoculated with 0·5 c.c. of platelets from Guinea-pig 4; result positive. Guinea-pig 32 inoculated with 2·5 c.c. of platelets from Guinea-pig 22; result positive.

The results with the blood of Case 3 tend to confirm the work of Kusama (1920) and Ségal (1922), which appeared to indicate that typhus virus in blood was found in a much greater concentration in a platelet emulsion obtained by centrifuging than in the other parts of the blood.

The leucocyte layer was not examined separately, since it is very difficult if not impossible to obtain the leucocytes in anything like purity.

The results with platelets from typhus guinea-pigs also confirm the observations of Ségal (1922).

Microscopic examination of whole blood, of leucocytes and platelets and of thick drops of platelet emulsion was carried out with material from the two human cases and from several of the guinea-pigs, but no fresh light was obtained on the question of the form taken by the virus in the blood. No reliable or constant variations from the normal could be established.

EXPERIMENTAL INFECTION OF LICE (*PEDICULUS HUMANUS*).

The lice used for these experiments were derived from a normal stock brought from England and fed on a healthy man. The lice after infection with typhus virus were fed on a typhus convalescent.

Feeding Normal Lice on Typhus Patients.

Lice in Box 5/2 were fed on Case 3 on 13 . ii . 22, which was stated to be the 13th day of the disease, and subsequently on a healthy person immune to typhus; result positive. Examination of the lice by dissection and smears showed *Rickettsia prowazeki* in one louse in the thread form on 23 . ii . 22 and in the minute form in one other louse on 2 . iii. Lice in Box 11/2 were fed on Case 4 for 11 days, beginning on 3 . iii, the day before the

temperature fell; result negative. These lice never showed any definite infection with rickettsia, and 11 of the lice from this box inoculated on 15 . iii into Guinea-pig 34 caused a febrile reaction of a not very convincing character. The small amount of infection discovered in these two boxes was probably due to the short time of feeding on a febrile patient in each case, and perhaps the late stage in the illness of each patient was a contributory factor. The fact that lice were demonstrably infected with rickettsia on Case 3 but not on Case 4 corresponds to the readiness with which guinea-pigs were infected by inoculations of the blood of Case 3 and to the relative difficulty of infecting with the blood of Case 4.

Experiments on the Inoculation of Lice per anum *with Emulsions of Platelets.**

Experiment 1.—15 lice in Box 5/1 were injected on 13 . ii . 22 with an emulsion of platelets from Case 3. The emulsion was made by including the platelets obtained from 1 c.c. of turbid plasma in 10 drops of salt solution. Result, positive. On 11 . iii (26th day) one louse was found to be heavily infected with rickettsia.

Experiment 2.—28 lice in Box 5/3 were injected with a minute portion of the platelets from 3 c.c. of turbid plasma from Guinea-pig 5 emulsified in 6 drops of saline. Result positive.

On 3 . iii (6th day) one louse was found to be slightly infected and on 19 . iii one louse was heavily infected with rickettsia; in all four lice showed infection.

Experiment 3.—36 lice in Box 5/4 were injected on 27 . iii with a platelet emulsion from Guinea-pig 4. Result positive.

8 . iii (9th day): A few threads of *R. prowazeki* were found in excreta.

9 . iii: 1 louse showed many *R. prowazeki.*

18 . iii: 2 lice, and 19 . iii: 4 lice showed *R. prowazeki.*

Experiment 4.—9 lice in Box 5/6 were injected on 14 . iii with an emulsion of the platelets from 3 c.c. of plasma of Guinea-pig 22 suspended in 8 drops of salt solution. Result positive.

22 . iii (9th day): Excreta showed many *R. prowazeki.*

23 . iii: The excreta which had been passed in the last 24 hours and showed *R. prowazeki* were injected subcutaneously into Guinea-pig 47 with positive result, and a similar result also followed the injection of the excreta passed on 24 and 25 . iii.

Experiment 5.—26 lice in Box 11/1 were injected on 2 . iii with platelets from Case 4. Result negative. No rickettsia were found in any of these lice, and many of them were infected with bacteria. The last two lice died 18 . iii. This result is in conformity with the difficulty experienced in infecting guinea-pigs with the blood of Case 4, and also the failure to infect lice by direct feeding on this patient.

Lice in two boxes were directly infected with *R. prowazeki* by means of an emulsion made from previously infected lice. The severe infection which it was found possible to obtain by injecting them *per anum* with material of

* The anal injection of lice was carried out exclusively by A. W. Bacot.

this kind enabled the thread forms of rickettsia and their intra-cellular position to be observed with great regularity.

Experiment 6.—22 lice in Box 5/5 were injected on 9 . iii *per anum* with an emulsion of a louse from Box 5/4 which had been infected with platelets from Guinea-pig 4. Result positive.

12 . iii : 1 louse showed indications of an early infection.

14, 15 and 16 . iii : Lice were found definitely infected with *R. prowazeki.*

15 . iii : The excreta were injected into Guinea-pig 33, and caused infection which was further transmitted.

Experiment 7.—35 lice in Boxes 5/7 *a* and *b* were injected on 17 . iii with an emulsion of a louse containing many rickettsia from Box 5/5. Result positive.

22 . iii : Excreta showed rickettsia definitely from Box *b*.

30 . iii : Excreta from (*a*) show some granules, possibly rickettsia, and those from (*b*) show swarms of rickettsia. This difference in the contents of the two boxes was accounted for by the fact that Box 5/7*a* had been kept at about 20–22°C., Box 5/7*b* at 32–33°C.

Remarks.—Weigl (1920) and Bacot (1922) described the technique of injecting *Pediculus humanus per anum,* and Weigl (1920) recorded observations on the infection of lice with *R. prowazeki* by injecting them with the leucocyte layer of citrated blood from typhus patients.

Bacot and Ségal (1922) showed that lice could be infected by the same method, using as virus an emulsion of platelets from typhus guinea-pigs. The separated platelets from human cases of typhus, however, have not as far as can be ascertained been used before to infect lice *per anum.* The matter is of some interest in connection with the claims of Kusama and Ségal that the virus of typhus is contained in or is closely adherent to the platelets in the blood.

It is worth noting that it was found on trial that a *thick* emulsion, *e.g.* the platelets from about 3 c.c. of blood emulsified in six drops of saline, was required to produce a severe and certain infection, and that it was best to inject the emulsion alone and not to combine it with a feed of whipped blood, since sometimes clotting occurred in such a mixture.

The above method of determining the presence of the virus of typhus, *i.e.* by examining lice for rickettsia after they have been injected with platelets, appears to be one of the best ways of establishing that an attack of fever in a guinea-pig following inoculation is really due to typhus virus; for though the nature of rickettsia bodies has been critically examined by various writers, few who have worked at this aspect of the subject would deny the constant association of typhus virus and *R. prowazeki* in lice.

Examination of Lice which had been Injected with Virus.

(a) *Microscopic examination for rickettsia.*—It will be seen from the last sections that of five attempts to infect lice with *Rickettsia prowazeki* by means of platelets from blood presumably containing typhus virus, four were successful, viz. one experiment (Box 5/1) with platelets from a human case, and three (Boxes 5/3, 5/4 and 5/6) with platelets from guinea-pigs. One experiment (Box 11/1) was unsuccessful with human platelets.

Of the three batches of lice (5/5, 5/7, 5/8) injected with material from the guts of three previously infected lice all became infected with *R. prowazeki.*

Of two attempts to infect lice with rickettsia by feeding them on human typhus patients one was successful and the other failed, but in both cases the possible infecting feeds were very few (see above).

(b) *Inoculation of emulsions of lice into guinea-pigs.*—The lice which had been artificially infected were examined in certain instances for virus by injecting them or their excreta subcutaneously into guinea-pigs.

(1) Eleven dead lice from Box 11/2 in which no rickettsia bodies could be found after feeding on Case 4 were inoculated on 15 . iii into Guinea-pig 34. Result doubtful; a slight febrile reaction.

(2) One dead louse from Box 5/5 showing abundant rickettsia bodies was inoculated on 18 . iii into Guinea-pig 40, with a positive result, and the infection was carried on by passage to two other guinea-pigs.

Inoculation of Excreta of Infected Lice into Guinea-pigs.

Experiment 1.—15 . iii . 22: Excreta of 20 lice in Box 5/5 showing many rickettsia forms were injected into Guinea-pig 33 with a positive result; a further passage with the blood of Guinea-pig 33 to Guinea-pig 45 produced a short but definite attack of fever.

Experiment 2.—16 . iii . 22: Excreta of 18 lice in Box 5/5 showing very many rickettsia bodies were injected into Guinea-pig 38 with a positive result, and passage in series to two other guinea-pigs (53, 48) produced typical reactions in each case.

Experiment 3.—19 . iii . 22: Excreta of 10 lice in Box 5/5 showing very many rickettsiæ were injected into Guinea-pig 41 with a positive result, and a further passage to Guinea-pig 54 caused a slight reaction.

Experiment 4.—23 . iii . 22: Excreta of 17 lice in Box 5/6 showing many rickettsiæ were injected into Guinea-pig 47 with a positive result and further passages in series to Guinea-pigs 67, 81 and 92 were also successful.

The above four experiments were made with excreta which were collected from the box of lice on the same day that they were injected. The next experiment was made with excreta which had been kept in a dry condition for some days; it was unfortunately the only one accomplished of a series of such experiments which it was intended to carry out.

Experiment 5.—5 . iv . 22: Excreta of not more than 16 lice in Box 5/6 which were collected on 24 and 25 . iii and kept 11 to 12 days were injected subcutaneously into Guinea-pig 72 with a positive result. The excreta were examined microscopically and found to contain abundant rickettsia-bodies. The temperature of Guinea-pig 72 was raised from the 11th to the 20th day from the inoculation and was 41°–41·1° C. for 3 days. The blood of this animal inoculated into Guinea-pig 82 produced a positive result with a maximum febrile reaction of 41·4° C.

Discussion of the Results of Experimental Inoculation of Infected Lice and their Excreta into Guinea-pigs.

The only doubtful result from the injection of louse material followed the inoculation of an emulsion of 11 lice from Box 11/2 which had fed on Case 4.

The guinea-pig inoculated (No. 34) had a rise of temperature of only about half a degree centigrade with a maximum of 40° C. The emulsion used did not contain any demonstrable rickettsiæ. It is noteworthy that each of the five specimens of excreta which contained numerous rickettsiæ produced a febrile attack in a guinea-pig such as is characteristic of a typhus infection. Though Nicolle, Blanc and Conseil (1914) found that the excreta of typhus-infected lice were regularly able to reproduce the disease in guinea-pigs, this has not been the experience of all workers. Rocha-Lima (1916) declared that he could not obtain infection with louse excreta, and Atkin and Bacot (1922) failed to infect animals with excreta of lice fed on a typhus monkey.

The most interesting positive result in this series was that obtained by injection of Guinea-pig 72 with excreta which had been kept at least 11 days in a dry condition. These excreta were shaken as a dry powder through the fine gauze cover of the box containing the lice into a sterile Petri-dish and were kept in a cupboard in the laboratory, remaining apparently quite dry. The result is noteworthy, since it has been generally held that drying rapidly destroys the virus of typhus. The lice affording these excreta were no doubt infected to a very high degree on account of the material and the method used for infection. Most experiments to test the resistance of the virus to drying have, however, been made with blood, in which the virus appears to exist in a much lower state of concentration. In the experiments recorded by Nicolle and his colleagues (1914) the excreta which proved virulent were apparently collected daily and inoculated on the same day that they were collected, and Nicolle (1922) mentions that he considers that excreta become inactive after a very short time—certainly within 8 days.

CONTROL EXAMINATION OF LICE WHICH HAD BEEN FED ON APPARENTLY HEALTHY PERSONS.

(1) The stock of normal lice brought from England and fed on a normal person was examined from time to time and was always found free from rickettsia.

(2) Normal stock lice in Box 2/1 were fed regularly on a recovered typhus patient who also nourished the experimental typhus lice. This control box was kept at 30° to 35° C. between the feeds, and the lice were examined occasionally but never showed any rickettsia bodies.

(3) Lice collected from apparently healthy persons, labourers, etc., living in Cairo.

Lice from 12 such persons were examined. In three of these only a few lice (2, 6 and 9 respectively) were examined, but from the remaining nine at least 12 lice were examined.

The lice from eight persons were quite negative as regards rickettsia, from two definitely positive results were obtained, and in nine lice from two other persons some rather uncertain bodies were found out of 34 lice examined (3 out of 22 and 6 out 12 lice). It must be remembered that doubtful results in a search for rickettsia are of very little value, since the *débris* of cells, etc., in films readily simulate a few scattered individual bodies.

No results have been recorded here as positive unless very definite rickettsia forms were present in large numbers.

The two persons from whom lice showed definite rickettsiæ were a blind man, "L," and woman, "K," who were of the poorest class and lived together. Lice were collected from them and examined twice and three times respectively.

From the man 53 lice were examined on one occasion (February 22nd) and 13 were found positive; on the other occasion 17 days later (March 11th) 18 lice were taken and all proved negative. From the woman 17, 8 and 24 lice were taken on three different days (February 19th, 21st and 22nd), and 1, 6 and 5 lice were found positive on these three occasions respectively.

The characters (staining, morphology and arrangement) of the rickettsia bodies found in lice from these two persons were apparently identical. They differed, however, in certain particulars from the forms found in trench fever and typhus. (See Plate.)

The bodies were usually very small and in general appearance resembled *R. prowazeki*. The staining with Giemsa was as a rule pinker and less purple than in the case of *R. quintana*, and though many of the forms were extremely minute and round or diplococcal in shape, there was often a tendency for them to form rods containing deeply stained dots, or short wavy threads which were sometimes uniformly stained. Most of the round or oval forms were no larger than the smallest *R. quintana*.

The most notable feature in their arrangement was the tendency to lie in large or small clumps, as if agglutinated, and even when fairly well emulsified small clumps of 6 to 10 individuals usually remained and few were quite isolated.

It was found possible to make a fairly good emulsion in distilled water but clumps formed again very rapidly in 0·85 per cent. salt solution.

The films of dissected lice showed evidence of the rickettsia being included in cells of the stomach.

This kind of rickettsia is very like that described and figured by Weigl (1922) under the name of *R. rocha-limæ*. The arrangement in clumps is an especially noticeable point of agreement, as is also the intracellular position.

Other features which Weigl describes as distinguishing *R. rocha-limæ* from *R. prowazeki* are, a much greater diversity of shape, good staining properties with dilute aniline dyes, ready transmission naturally from one louse to another in the same box and even through the medium of boxes, etc., soiled with excreta, absence of virulence for guinea-pigs and a very low virulence for lice when injected *per anum*. The rickettsia which was found in Cairo agreed with Weigl's species in the absence of, or very feeble virulence for guinea-pigs, but not in the other particulars, except the clumped arrangement. The shape and size, though somewhat variable, did not cover nearly so wide a range as is often the case in *R. prowazeki*. Methylene blue or dilute fuchsin stained both species very feebly and about equally: normal lice in captivity did not become infected by contact, and *per anum* injection of both kinds into lice caused a rapid infection of the gut cells and early death of some individuals, but injection with the new variety appeared to be rather more fatal than with *R. prowazeki*.

The infection was passed on to six boxes of normal lice by injecting them *per anum* with emulsions of the gut of lice found naturally infected with the new kind of rickettsia.

Box 6/1: 24 lice injected from lice taken from "K"; large numbers of rickettsia were found in some of the lice on the 14th day from injection.

Box 6/2: 12 lice injected from lice in 6/1; positive on 7th day.

Box 7/1: Lice injected from a louse taken on "K"; positive on 7th day.

Box 7/2: Lice injected from a louse in Box 7/1; positive on 7th day.

Box 7/4: Lice injected from a louse in Box 7/2; positive 3rd day.

Box 7/6: 20 lice injected from a louse in Box 7/4; the excreta contained many rickettsia of characteristic appearance on the 4th and 6th days.

Source of Infection of the Lice with the Cairo Rickettsia.

No evidence was obtained that the infection passed naturally from louse to louse by copulation or by contact in the same box, but experiments in this direction were not completed.

Normal lice from Box 7/3 were immersed in a thick emulsion of rickettsia from lice of Box 7/2, but did not become infected.

Evidence of heredity in the louse was sought by examining the eggs of infected lice and first skin larvæ caught at the same time as infected adult lice.

The eggs on dissection showed no definite rickettsia, but several larvæ contained small groups of bodies believed to be rickettsia.

The attempt was made to determine whether the infected lice caught on "K" and "L" had derived the rickettsia from the blood of their hosts. Normal lice were accordingly fed regularly on these two persons.

(1) 22 . ii . 22: Uninfected lice in Box 6/3 were fed once daily on "K." 22 . iii . 22: No infected lice had been detected.

(2) 15 . iii . 22: 40 normal lice in Box 6/4 were fed on "K" daily but no infection could be found by 22 . iii . 22.

(3) 22 . ii . 22: Normal lice in Box 9/1 were fed daily on "L" and kept at 26°C. 28 . iii . 22: No rickettsia had been found so far.

(4) 27 . ii . 22: Some lice originally collected on "L" were put in Box 10/1 and daily fed on him, but up to 28 . iii . 22 no rickettsia could be detected in the excreta or dead lice.

It seems clear, therefore, that either the lice taken on "K" and "L" had not become infected by feeding on these persons and derived their rickettsia from some other source, or else that one or both of the hosts had recently harboured rickettsia in the blood but had recovered from the infection before the feeding experiments were begun.

The relation of the new species found in Cairo in lice from "K" and "L" to the previously described species in *Pediculus humanus* must remain at present uncertain. However, the possibility must not be overlooked that it may be a non-virulent variant of *R. prowazeki*, and the differentiating characters are not so definite as to exclude this possibility.

The experiments made to test the virulence of this form of rickettsia give very little encouragement to the hypothesis that a strain of *R. prowazeki* was present in these lice.

Though most of the inoculations were without effect, the reactions of two guinea-pigs—18 and 56—which occurred at the first passage from guinea-pig to

guinea-pig were sufficient to encourage further work in this direction when another opportunity occurs.

CONCLUSIONS.

(1) The infectivity for guinea-pigs of an emulsion of platelets (nearly quite free from other blood cells) from a human case of typhus was high considering the small amount of blood material which it contained.

(2) The injection of lice *per anum* with an emulsion of platelets from a human case of typhus caused severe infection of the lice with *R. prowazeki.*

(3) The injection of lice *per anum* with emulsion of platelets from typhus guinea-pigs produced an infection of the lice with *R. prowazeki,* as previously shown by Bacot and Ségal.

(4) Lice infected with *R. prowazeki* were infective for guinea-pigs whether an emulsion of the lice themselves or of their excreta was used for the inoculation.

(5) The excreta of infective lice will remain active as a dry powder at laboratory temperature for 11 days.

(6) 204 lice from 12 apparently healthy persons were examined for rickettsia. 84 of these from 10 persons were negative (except for a few uncertain results).

(7) In films of 25 out of 120 lice taken from two apparently healthy persons who lived together rickettsia bodies were found.

(8) Lice from the two last-mentioned persons were only found to be infected during a period of four days, and lice taken from them subsequently were free, nor did fresh lice become infected by feeding on them.

(9) The rickettsia found in lice from these two persons resembled in some respects *R. rocha-limæ* and *R. prowazeki.* One of the most obvious special features was the occurrence of the minute forms in large dense clumps—an unusual arrangement in the case of *R. prowazeki* and *R. quintana,* but characteristic of *R. rocha-limæ.*

NOTE ON THE CAUSE OF LABORATORY INFECTIONS WITH TYPHUS.

The exact manner in which laboratory workers have become infected with typhus fever has not always been clear. It has usually been accepted that an actual transference of at least one infected louse from the sick to the healthy has occurred. In many cases no doubt a risk of acquiring infected lice has been incurred by contact with patients.

In the present instance no such opportunity occurred up to the time when the first laboratory infection (A. W. B., onset March 26th) was acquired, since neither of the two workers had recently seen a case of typhus nor been to the fever hospital. They had merely received infective material at the laboratory and had propagated the virus in guinea-pigs and lice. The second person attacked (J. A. A., onset April 5th) had visited A. W. B., but only in a perfectly clean hospital ward. There is no reason to suppose that any infected lice had escaped from boxes in the laboratory; indeed, the reverse is practically certain.

It has been widely held that the disease is usually communicated by the

actual bites of lice, but very few recorded observations have been made to test this hypothesis in such a way as to exclude infection by means of the excreta of the lice. Atkin and Bacot (1922) made two careful attempts to infect macacus monkeys by feeding infected *Pediculus humanus* on them, and one similar attempt to infect a guinea-pig, taking care that the infection should not take place by inoculation of the excreta. None of these three animals became infected. There is no microscopic evidence suggesting that rickettsia might be transferred from the louse by the act of biting, nor can these bodies be demonstrated in the fore-part of the gut, mouth parts, or salivary glands.

The infectivity of excreta had become impressed on the workers in the present investigation by their proved virulence for guinea-pigs which were inoculated on March 15th and 16th, and a special investigation as to the dose and keeping properties of infective louse excreta had been planned and begun when A. W. B. became ill.

That the virus in louse excreta can readily enter the body through abrasions or slight wounds is very probable, and this has been shown to happen in the analogous case of trench fever. An unsuccessful attempt to infect a guinea-pig with the excreta of lice fed on a typhus monkey has been recorded by Atkin and Bacot (1922), but it is most probable that the infection is much more readily produced in man than in animals.

Töpfer (1916), in speaking of the death of Schüssler following a laboratory infection with typhus, says that in all probability the virus was introduced into the eye or mouth by the finger. He states, moreover, that Schüssler had an inflammatory affection of the lids, which indicated a possible place for the accidental inoculation to take place.

On the whole, then, it seems probable that excreta of infected lice are one, if not the chief source of laboratory infections with typhus, but of course other means of infection cannot be ignored, such as a prick with an infected dissecting needle.

Thanks are due to Dr. Charles Todd, the Director, and all the staff of the Public Health Laboratories, Cairo, for their courtesy and help; and also to Dr. Sami Sabongi, Medical Officer in Charge of the Fever Hospital, Abbassia, for his assistance in providing us with infective material, and help in other ways.

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DESCRIPTION OF PLATE.

Drawings made from stained films of emulsions from the guts of lice infected with *Rickettsia sp.?* found on two apparently healthy persons in Cairo.

FIG. 1.—From gut of dead louse from Box 7/1 injected with emulsion of the gut of another infected louse on 23 . ii . 22; film made 6 . iii . 22—11th day.
FIG. 2.—From gut of louse of Box 7/2 injected 4 . iii . 22 with emulsion of a louse of Box 7/1; film made 13 . iii . 22—9th day.
FIG. 3.—Film from gut of louse of Box 7/2; killed, and film made 7 . iii . 22—4th day.
FIG. 4.—Low-power view of the same film from which fig. 3 was taken, showing characteristic arrangement in clumps.

FIGS. 1–3 approximately × 1000.

THE BRAIN AND IMMUNITY REACTIONS TO JENSEN'S RAT SARCOMA.

W. S. LAZARUS-BARLOW, M.D., F.R.C.P., AND R. H. PARRY, M.B., B.S.

From the Cancer Research Laboratories, Middlesex Hospital, London.

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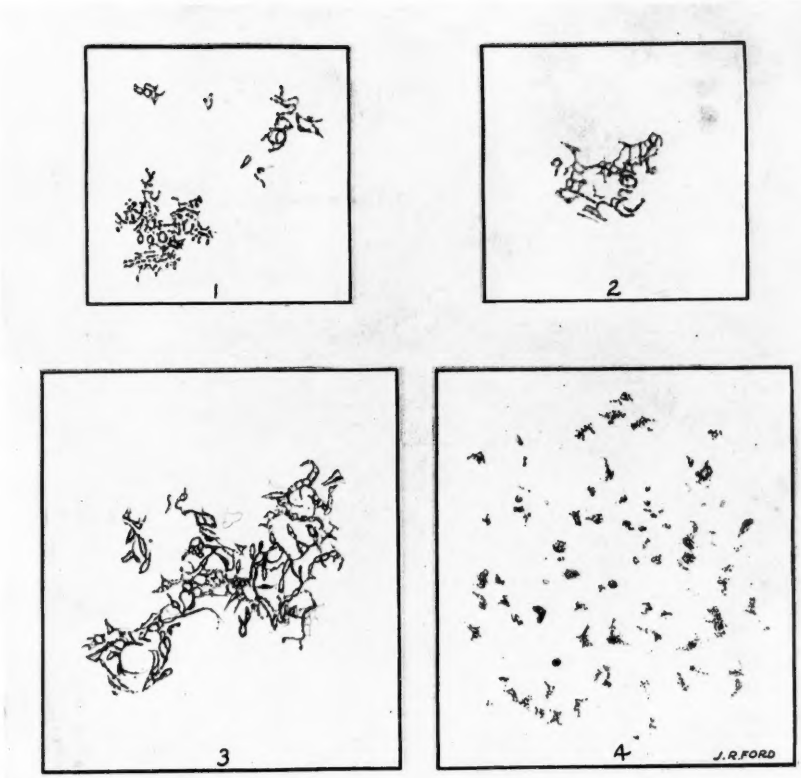
IN the course of experiments with Jensen's rat sarcoma, which consisted in simultaneous inoculation of grafts into various tissues of the body, a difference between rats in which the brain was inoculated and those in which it was not inoculated seemed to manifest itself. Thus of 25 animals in which brain inoculation was omitted, only 5 (20 per cent.) showed absence of measurable growth at the site of subcutaneous inoculation, whereas 11 animals in which brain inoculation was performed showed absence of measurable growth at the site of subcutaneous inoculation in 5 (45 per cent.), and a similar absence of growth in other organs into which growths were made. Though grouped



Arkwright and Bacot.

together for brevity these results were not obtained on a single occasion, and though they might be referable to injurious effects of the cerebral operation upon general nutrition, it seemed desirable to test the matter under stringent conditions owing to its possible bearing upon the production of an active immunity in malignant disease.

A tumour was taken, which by synchronous experiment was seen to grow rapidly and take subcutaneously in 100 per cent. of cases, and the following experiment was made:

A. In 20 normal rats brain puncture without introduction of graft was performed.

B. In 20 normal rats grafts were introduced into the brain.

C. In 20 normal rats grafts were introduced into the right flank.

Seven days later the 15 survivors of A, the 16 survivors of B and the 20 survivors of C were inoculated into the left flank with a tumour which by synchronous experiment was seen to grow rapidly and take subcutaneously in 95 per cent. of cases. On the 10th day after this second inoculation the left sides of all the animals were examined for growth, and it was found that in A 12 (80 per cent.), in B 9 (56 per cent.), in C 15 (75 per cent.) showed measurable subcutaneous tumours. Taking the volume of a tumour as being represented by the value (length × breadth) $\frac{3}{2}$, the mean amount of tumour per rat of series A was 290 c.mm.; of series B, was 141 c.mm.; of series C, was 148 c.mm.; and of the controls, was 228 c.mm.

At the end of 5 weeks from the first inoculation, 3 rats in series A and 3 in series B were living, healthy and tumour-free, whereas all of the animals in series C were dead as the result of tumour growth.

The Jensen tumour selected took so readily and its growth was so rapid even in the brain that a hypothetical immunity mechanism was placed under the most adverse circumstances, for it needs but a small tumour (*e.g.* 5 mm. in diameter) within the skull cavity to kill the animal by pressure. Hence it was determined artificially to lower the vitality of the tumour used for inoculation in the hope of allowing more time for the brain to respond to the inoculation or to accommodate itself to a growing graft. With this object a tumour which in synchronous experiment on the 10th day had taken subcutaneously in 92 per cent. of cases and yielded on an average 476 c.mm. of growth per rat of the series, was exposed to X-rays under standard conditions for 25 minutes.* On the tenth day after subcutaneous inoculation grafts of this irradiated material had taken in 85 per cent. of cases and yielded on an average 244 c.mm. per rat of the series. The original experiment was repeated under the new conditions. Twenty normal rats (series D) received intracerebral grafts of the irradiated tumour, and 20 normal rats (series E) received irradiated tumour in the right flank. Seven days later the 17 survivors of D and the 20 survivors of E received into the left flank grafts of a subcutaneous tumour which during its whole existence had grown rapidly and at exponential rate,

* For all the irradiations carried out in this investigation we are indebted to Miss Gladwys Scott. The "standard conditions" were: Distance of the tumour from the anticathode of a Coolidge tube 30 cm., alternative spark-gap 5 cm. between spheres 5 cm. in diameter, output of bulb checked every minute by electroscopic readings. Increased impairment in vitality of graft by irradiation was aimed at by giving 25 minutes, 35 minutes and 55 minutes' exposure respectively.

and by synchronous experiment was shown to take subcutaneously in 80 per cent. of cases and yield on the tenth day a mean value of 262 c.mm. per rat of the series. On the tenth day after the second (subcutaneous) inoculation it was found that of the primarily brain-inoculated animals (D), 13 (76 per cent.) showed measurable subcutaneous tumours with a mean volume of 351 c.mm. per rat of the series, while the primarily flank-inoculated animals (E) showed 70 per cent. of measurable secondary subcutaneous tumours with a mean volume of 346 c.mm. for each rat of the series. As said above, the control value was 262 c.mm. At the end of 5 weeks from the first inoculation all the animals of series D and E were dead, while 20 per cent. of the controls were living and without tumour.

Examination of the results in series D and E, therefore, shows that both primary brain-inoculation and primary flank-inoculation with tumour irradiated under standard conditions for 25 minutes actually led to a greater volume of tumour, on test-inoculation, per rat of the series than in the controls, while any reduction in percentages of takes was negligible. Consequently it appears that primary inoculation of tumour irradiated with this intensity has favoured subsequent growth in the subcutaneous tissue instead of impairing it as was anticipated.

Since it appeared from series D and E that a local delay of growth (*cf.* below) might be associated with a general increase of sensibility, it was determined to increase further the degree of irradiation. Hence series F and G with tumour irradiated for 35 minutes under standard conditions, and series H and I with a tumour irradiated for 55 minutes under standard conditions, were carried out on identical lines with those already described. The test (subcutaneous) inoculation was made with a tumour which synchronous experiment showed to produce measurable tumours in 85 per cent. of cases, and to yield on the tenth day a mean value of 766 c.mm. per rat of the series. The results on the tenth day after test inoculation were as follows: In F measurable subcutaneous tumours were found in 17 out of 20 survivors (85 per cent.), and the mean volume of tumour per rat of the series was 590 c.mm.; in G, 13 out of 19 survivors (68 per cent.) showed measurable tumours and the mean volume of growth was 331 c.mm. per rat of the series; in H, 15 out of 21 survivors (71 per cent.) showed measurable tumours and the mean volume of growth was 476 c.mm. per rat of the series; while in I, 9 out of 19 survivors (47 per cent.) showed measurable tumours and the mean volume of growth per rat of the series was 178 c.mm. At the end of 5 weeks from the first inoculation, in series F, 1 rat was living, healthy and tumour-free; in series G there were 2; in series H there were 6; and in series I there was 1.

BEARING OF EXPERIMENTS UPON THE QUESTION OF *GENERAL* RESISTANCE.

For purposes of comparison, the mean amounts of tumour per rat of the various series may be expressed as percentages of the corresponding mean amounts of the controls. If this be done the values set forth in Table I are obtained.

TABLE I.—*Mean Volumes of Tumour on the Tenth Day in Test (Subcutaneous) Inoculations after a Preliminary Treatment of the Animal by Intra-cerebral or Subcutaneous Inoculation.*

(The values are expressed as percentages per rat of series of the corresponding mean volumes in the controls.)

Site of preliminary inoculation.	Treatment of tumour used for preliminary inoculation			
	Nil.	25 minutes' irradiation.	35 minutes' irradiation.	55 minutes' irradiation.
Brain	62	134	77	62
Subcutaneous (flank)	65	132	43	23

From these values it appears (1) that a previous inoculation with non-radiated tumour—whether intra-cerebral or subcutaneous—leads to a certain amount of general resistance to growth of a subsequent inoculation; (2) that a previous inoculation with tumour that has received a 25 minutes' dose of X-rays—whether intra-cerebral or subcutaneous—leads to a great diminution of general resistance to growth of a subsequent inoculation; (3) that previous inoculation with tumour that has received a 35 minutes' or a 55 minutes' dose of X-rays—whether intra-cerebral or subcutaneous—leads to a diminishing susceptibility to growth of a subsequent inoculation, which in the case of preliminary subcutaneous inoculation passes into the acquisition of a definite amount of increased general resistance or immunity. The difference in degree between the effect of previous intra-cerebral and that of previous subcutaneous inoculation with the 35 and the 55 minutes' irradiation of the graft we ascribe to the injurious effects of cerebral puncture, for an examination of Series A will show that cerebral puncture alone induces a certain amount of diminished general resistance to growth of a subsequent inoculation, and therefore counteracts to some extent the immunity conferred by irradiated tumour.

Examination of the numbers of healthy and tumour-free animals in the various series at the conclusion of the experiments (5 weeks after the initial inoculations) bears directly upon the question under discussion. They are given in Table II. The number of animals theoretically expected to be living at the end of the experiment was determined from the percentages of "takes" in the control animals for the different samples of tumour used as grafts. Thus, if 100 rats were inoculated with an 80 per cent. tumour and a week later with a 90 per cent. tumour, 80 rats would ultimately succumb to the first inoculation, and $\frac{90}{100}$ of the remaining 20 rats (*i. e.* 18) would ultimately succumb to the second inoculation. Hence at the end of the experiments, theoretically only 2 animals would be living.

From Table II it appears that a greater degree of general immunity is conferred upon the animal by a preliminary intra-cerebral inoculation with non-radiated tumour or tumour irradiated under standard conditions for 55 minutes than by a preliminary subcutaneous inoculation with similar material. The effect produced by intra-cerebral inoculation upon general resistance is further indicated by the behaviour of the test inoculations in the 10 rats that survived. In 4, the graft failed from the first; in 6, a tumour, often of a considerable size (*e. g.* 12 × 15 c.mm.), was formed, but subsequently underwent complete retrogression or degeneration.

TABLE II.

Series.	Site of preliminary inoculation.	Treatment of graft in preliminary inoculation.	Treatment of graft in test inoculation (subcutaneous).	Living, healthy and tumour-free animals at end of experiment. Theoretical.	Actual.
B	Brain	*Nil*	*Nil*	None	19 %
C	Flank	,,	,,	,,	None
D	Brain	25 minutes' irradiation	,,	,,	,,
E	Flank	,, ,, ,,	,,	,,	,,
F	Brain	35 ,, ,,	,,	4 %	5 %
G	Flank	,, ,, ,,	,,	,,	11 %
H	Brain	55 ,, ,,	,,	9 %	29 %
I	Flank	,, ,, ,,	,,	,,	5 %

BEARING OF THE EXPERIMENTS UPON THE QUESTION OF LOCAL RESISTANCE.

The ultimate percentage of "takes" shows no difference according as the graft is intra-cerebral or subcutaneous. But if the amount of growth as the result of primary inoculation be considered it is seen that locally a great difference manifests itself. Thus, in spite of the fact that all primarily brain-inoculated animals in Series D (25 minutes' irradiation) ultimately died from cerebral tumour, yet on the twentieth day after inoculation 50 per cent. of them were living and showed no evidence of a brain lesion, whereas all the primarily flanked inoculated animals (Series E) showed a tumour at the site of inoculation so large that if one-tenth had been present within the brain the animals would have been moribund.

So also on the twentieth day after inoculation 75 per cent. of the primarily brain-inoculated animals in Series F (35 minutes' irradiation) and 100 per cent. in Series H (55 minutes' irradiation) were living and showed *no* evidence of cerebral lesion, whereas 77 per cent. and 43 per cent. of the primarily flank-inoculated animals in the corresponding Series G and I showed considerable masses of growth.

Similarly with non-irradiated grafts (Series B and C), 50 per cent. of the primarily brain-inoculated animals were living and showed no sign of a brain lesion on the twentieth day after primary inoculation, whereas all the primarily flank-inoculated animals showed a large local tumour. Hence it is clear that whether irradiated or not the early stages of an intra-cerebral graft are distinctly impeded as compared with the early stages of a graft made into the subcutaneous tissue. Delay of a graft to grow may depend upon (*a*) absence of suitable growth conditions or (*b*) presence of an antagonising mechanism, and on this question the experiments quoted above give no information. But a certain amount of information is afforded by histological examination of the cerebral grafts and tumours.

HISTOLOGICAL RESULTS.

Microscopical examination of the brain was made in 130 cases, of which 19 are excluded because the animals died shortly after operation. The remainder

consisted of brain puncture without inoculation of graft, 14 cases; intra-cerebral inoculation of non-radiated graft, 25 cases; of graft irradiated for 25 minutes, 18 cases; of graft irradiated for 35 minutes, 20 cases; of graft irradiated for 55 minutes, 17 cases; intra-cerebral inoculation of non-irradiated grafts into immune animals, 11 cases; and normal brains of immune rats, 6 cases.

The results of inoculation at death of the animal are shown in Table III:

TABLE III.

Graft.	Active growth present.	Degenerated growth present.	Track alone present.	Total.
Non-irradiated.	18	6	1	25
25 minutes X-rays.	17	0	1	18
35 ,, ,,	18	0	2	20
55 ,, ,,	9	6	2	17

In those cases in which active growth was present, the histological characters of the cells, the number of mitoses, the vascularity, the absence of degeneration resembled these features in actively growing subcutaneous grafts, but the growing edge offered a considerable difference. In subcutaneous tissue the tumour resulting from a graft of Jensen's rat sarcoma shows few finger-like processes at the periphery or none at all; in the brain, on the contrary, such a tumour shows extensive finger-like invasion of the adjacent tissue except where it reaches the meninges or lateral ventricle. A very few lymphocytes or small mononuclear cells may lie in advance of the growing edge, especially where it reaches the meninges, but where brain substance is being invaded there is a remarkable absence of adventitious cells.

It follows from this description, which agrees in all essential points with the findings of Da Fano (1912), that the developing tumour in brain offers no evidence that the local nutritive conditions for growth are wanting.

In degenerating or fully degenerated grafts, or in tracks whence the graft or tumour has disappeared, a different picture is presented. Cells with special features are almost invariably found—often in great numbers. These cells have a diameter of 12 microns or more, are often well-defined, possess an excentric, round or oval, pale, diffusely staining, nucleolated nucleus, and a sparsely granular cytoplasm that stains faintly with pyronin. The nuclei differ little from one another but the cytoplasm appears to undergo disintegration readily, so that the outlines of the cells may be ill-defined and naked nuclei may lie in a granular *débris*. The cells may be discrete or may be aggregated into a veritable tissue which somewhat resembles the cortical substance of adrenal. Such an accumulation of cells surrounds a focus of degeneration more or less completely and separates it from the brain substance. A few lymphocytes or small mononuclear cells may be interspersed amongst the large special cells under consideration, but polynuclear leucocytes are absent. In the form which has been described above the finely granular cytoplasm does not contain any ingested material, but other cases occur in which the cytoplasm of large—apparently identical—cells is

crammed with *débris* of red blood-corpuscles. This occurs notably in the track of a puncture without inoculation or a track after disappearance of the graft. Where the amount of blood *débris* is small the nucleus of the cell is distinguishable, but usually it cannot be detected (Fig. 1).

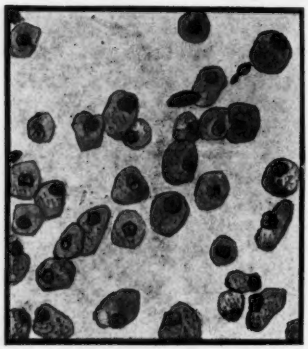


FIG. 1. × 600.

We are not in a position to affirm the nature of these cells. In many respects they bear resemblances to the so-called "macrophages" and may be associated with typical plasma-cells. They give no lipoid reaction, they are never found within blood-vessels, they are not altered tumour-cells, since they occur in the track of a brain puncture without inoculation of graft,* they are not found earlier than the fifth day after inoculation, they are usually associated with degeneration of the graft or with healing, but in two cases we have found them in advance of the growing edge of a graft of lowered vitality (35 and 55 minutes' irradiation respectively). The fact that we have found them in advance of a growing edge suggests to us that they are not merely a sequel to degeneration, but form part of an active reaction to tumour growth whereby degeneration of that growth is brought about.

CONCLUSIONS.

In the case of Jensen's rat sarcoma and the normal rat:

(1) Intra-cerebral inoculation of non-radiated or heavily-radiated grafts induces a subsequent increased general resistance to inoculation which is greater than that induced by inoculation of similar material subcutaneously.

(2) Intra-cerebral or subcutaneous inoculation of less heavily irradiated grafts induces a subsequent increased general sensibility to inoculation.

(3) The growth of a graft within the brain is restrained and delayed as compared with that of one in the subcutaneous tissue. This restraint and delay results in a greater frequency of degeneration and disappearance of the intra-cerebral graft, perhaps after a preliminary period of growth.

(4) The degeneration of an intra-cerebral graft or growth in the normal (and in the immune) rat is associated with the presence of large special cells which are derived from the host, and apparently form part of the process whereby that degeneration is brought about.

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* Cells, apparently identical, were found by the Macklins (1920) in repair of brain after aseptic lesions produced by hot or cold needles.

THE RELATIVE CONCENTRATION RATIOS OF SOME CONSTITUENTS OF THE URINE.

I.—THE DISTRIBUTION BETWEEN PLASMA AND CORPUSCLES, AND THE CONDITION IN THE BLOOD, OF UREA, CREATININE, INORGANIC PHOSPHATE AND URIC ACID.

S. W. F. UNDERHILL,

Research Scholar of the British Medical Association.

From the Department of Physiology, St. Bartholomew's Hospital, London.

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DURING the last few years observations have been accumulating, which suggest that the "filtration reabsorption" theory of renal secretion, put forward with a large body of evidence in its favour by Cushny (1917), in his monograph, requires modification. Investigations have been along two main lines: first, a direct examination of the passage of substances through the kidney of the frog; secondly, a larger number of observations of a more indirect nature on both man and animals.

Accepting the division of the constituents of the urine into the two groups of "threshold" and "no-threshold" bodies, it is clear that if two of the latter group simultaneously present in the blood are filtered off through the glomeruli and subsequently undergo concentration in the tubules from the absorption of water, the degrees to which they are concentrated should be the same in each case. This "concentration ratio" can be best expressed as the ratio of the quantity per cent. in the urine to the quantity per cent. in the plasma (MacLean, 1921). It is only the amount in the *plasma* which is available for filtration through the glomeruli, and then only if the substance in question is present in a "free," *i.e.* in a dialysable condition. This involves a consideration of the condition and distribution in the blood of the substances used in this work. Urea, inorganic phosphate, creatinine and uric acid were chosen.

The first three are described as true "no-threshold" bodies, while uric acid occupies a somewhat intermediate position between the no-threshold and threshold groups (Cushny, 1917). The selection of these substances was partly governed also by the availability of methods of analysis applicable to small quantities of blood and urine. Furthermore, except in the case of uric acid (Jones, 1920), these bodies undergo no further change in the animal organism. Marshall and Davis (1914) have shown this experimentally for urea; Cathcart (1916) and Barnett and Addis (1917) found, however, an increase in the ammonia of the tissues after the injection of urea.

A recent paper by Wu (1922) on the distribution of various substances between plasma and corpuscles in man gives a review of the previous literature.

He obtained an even distribution of urea, thus confirming earlier investigators, but found less creatinine in plasma than in corpuscles. The previous work of Wilson and Plass (1917), and Hunter and Campbell (1918), however, had shown an equal distribution for this substance also: and Wu states that the method used (precipitation method of Folin and Wu, 1919) may give too high results when applied directly to corpuscles. The former investigators estimated creatinine in whole blood and plasma and so obtained the amount in the corpuscles by calculation; they also allowed for the errors in the picric acid precipitation method, used on whole blood; Wilson and Plass, moreover, drew their conclusions from estimations performed after precipitation of proteins by heat coagulation.

As regards inorganic phosphate, Bloor (1918) found that there was twice as much in the corpuscles as in the plasma, but Zucker and Gutman (1922) observed a uniform distribution. The ease with which organic phosphorus is hydrolysed in acid solutions, such as are frequently used for protein precipitation, renders the determination difficult.

As regards the uric acid, which was used in a few experiments only, Wu (1922) found a higher value for the plasma than for the corpuscles; Theis and Benedict (1921) found an equal distribution in half of the cases examined, and in the majority of the remainder a higher amount in the plasma; Morris and MacLeod (1922) obtained about twice as much in the serum as in the corpuscles.

Turning to the question of the condition of these substances in the blood, Cushny (1919) has shown that urea and phosphate are completely dialysable, and should, therefore, be filterable through the glomerular membrane. Creatinine is also assumed to be present in the "free" state. As regards uric acid, it is suggested that a combined form exists in blood as well as the free acid, or free urate, but opinions differ as to how far the combined form reacts with the Folin uric acid reagent or its modifications (Benedict, 1922; Morris and MacLeod, 1922). Guillaumin (1922) states that the plasma contains mostly the free form and the corpuscles chiefly the combined; it has been suggested that part of this combined form is a combination with a pentose such as has been found in ox blood by Davis and Benedict (1921). Richter-Quittner (1921), however, states that uric acid is completely dialysable.

In experiments on the function of the kidneys, the simplest method, it might be thought, would be to use estimations made on the plasma. Apart altogether from the fact that the majority of the observations mentioned above have been made on man, while in these experiments it was proposed to use animals, Falta and Richter-Quittner (1921) have stated that, among other substances, urea, inorganic phosphate and sugar are not present in the corpuscles under physiological conditions, and do not enter them if precautions are taken to prevent injury to them when the blood is drawn.

Brinkman and Van Dam (1919) had shown previously that the corpuscles of frog and man are impermeable to glucose; and Ege (1921) has confirmed this conclusion for some animals, but not for man, and Van Creveld and Brinkman (1921) for man and rabbit. Apart from the discrepancies between the results of the different investigators, they also fail to agree as to what it is which causes the corpuscles to become permeable. Thus Brinkman holds that

the commencement of clotting alters the permeability, while Falta states that *serum* has the same amount as plasma if defibrination is properly performed. The latter investigator also considers that corpuscles are permeable to sugar *in vivo*, since they use it as a food; but presumably they do not store it, or carry it, in the same way as plasma, and so contain none when blood is drawn with proper precautions.

If, therefore, under ordinary conditions of drawing blood into potassium oxalate, substances enter the corpuscles from the plasma, while *in vivo* they remain entirely in the latter, it would be necessary in these experiments to estimate them in whole blood, and with the aid of a hæmatocrit reading, calculate the amounts in the *plasma*, and so the amounts which are available for kidney secretion.

It seemed of interest, then, to inquire as to the distribution of these substances between plasma and corpuscles, and a series of experiments was undertaken with this object in view.*

METHODS.

Since the kidney concentration experiments were performed on cats, only the results obtained with these animals will be mentioned here.

The animals were anæsthetised with ether and bled from an artery through a paraffin-lined cannula, the blood being collected in paraffin-lined centrifuge tubes, containing the requisite amount of anti-coagulant; the sample (or a portion of it) was then immediately centrifuged. The estimations were performed on whole blood and plasma; the amounts in the corpuscles were calculated from hæmatocrit readings obtained from the same or parallel samples of blood.

The methods used for the analyses were:

For urea, creatinine and uric acid, the colorimetric methods of Folin (1921); the proteins of the plasma were precipitated by tungstic acid and the estimations performed on the filtrate: the urease method was used for urea, followed, in the plasma filtrate, by aspiration into standard acid and Nesslerisation, and, in urine, by direct Nesslerisation; the NH_3 in urine was estimated by aspiration into acid and Nesslerisation.

For inorganic phosphate the colorimetric method of Bell and Doisy (1920) was used, with the precautions suggested by Myers and Shevky (1921).

Special experiments were only done for the distribution of urea and creatinine; sometimes blood was taken at the end of an experiment in which two or more of the substances under investigation had been injected into the circulation and the distribution determined. Various substances were tried as anti-coagulants, especially sodium oxalate and sodium citrate, since Falta and Richter-Quittner (1921) state that they cause less damage to the corpuscles than potassium oxalate. Sodium citrate, however, was unsatisfactory, causing hæmolysis.

RESULTS.

The results obtained are set out in Table I.

These confirm, on the whole, the conclusions reached by previous

* This piece of work was undertaken in conjunction with Dr. George Graham. With his permission I quote the results as far as concern the subject of this paper.

investigators, and suggest that although there may be variations in the distribution of urea and creatinine in either direction, yet there is no regular departure from equality of distribution in either instance.

Inorganic phosphate, and possibly uric acid, show larger amounts in plasma than in corpuscles; this may have been because the conditions of experiment were somewhat different, these substances having been purposely injected into the blood-stream some time beforehand. Creatinine and urea appear to behave similarly after injection, as is seen in experiments No. 13 and 16.

TABLE I. *(PART I)*
DISTRIBUTION BETWEEN PLASMA AND CORPUSCLES.

EXPERIMENT N°	ANTICOAGULANT	CORPUSCLES VOL %	BLOOD. mg PER 100 c.c.	PLASMA. mg PER 100 c.c. = P	CORPUSCLES. mg PER 100 c.c. = C (CALCULATED)	$\frac{P}{C}$
			UREA			
3	C	37	63·3	69·5	52·7	1·3
	B	38		70·4	51·6	1·4
4	A	42		68·9	38·4	1·2
	B	39	62·5	66·0	56·9	1·2
	C	37		67·7	53·8	1·25
5	C	35	50·9	52·2	48·6	1·1
6	C	37·5	84·3	86·2	81·1	1·1
7	C	33	39·9	36·8	46·4	0·8
12*	C	20	120	110	160	0·68
13*	C	29·5	101	103	96	1·07
16*	C	31	107	117	84·8	1·4
			CREATININE			
3	C	37	1·2	1·0	1·5	0·67
	B	38		1·1	1·4	0·78
4	A	42		1·1	1·3	0·85
	B	39	1·2	1·2	1·2	1·0
	C	37		1·2	1·2	1·0
12*	C	20	2·9	3·0	2·5	1·2
13*	C	29·5	1·9	2·4	0·7	3·4
16*	C	31	4·3	4·9	2·9	1·7
			INORGANIC PHOSPHATE			
12*	C	20	8·9	9·9	5·0	1·98
13*	C	29·5	7·7	8·5	5·8	1·5
16*	C	31	15·5	17·9	10·3	1·7
			URIC ACID			
13*	C	29·5	0·96	1·1	0·64	1·7

A = 0·1g SODIUM OXALATE PER 100 c.c. BLOOD
B = 1·0g SODIUM CITRATE " " "
C = 0·2g SODIUM OXALATE " " "
* SIGNIFIES THE SUBSTANCE INVESTIGATED HAD BEEN PREVIOUSLY INJECTED INTO THE BLOODSTREAM.

Iversen (1921) found that injected Na_2HPO_4 gradually entered the corpuscles of the rabbit, becoming organically combined, if excretion was prevented by nephrectomy. Theis and Benedict (1921) observed that uric acid added to blood remained in the plasma in 70 per cent. of the cases, in 30 per cent. becoming equally distributed. In the one experiment in which the uric acid distribution was determined, slightly more was present in the plasma as compared with the corpuscles. In my experiments it sometimes happened that the whole blood estimation was performed on blood, a portion of which was not subsequently centrifuged; in this way there might be an actual small variation between the amount originally present in the whole blood and plasma samples which would account for an apparent uneven distribution between plasma and corpuscles. This was sometimes unavoidable owing to the small quantities of blood available, and it is known that dilution of the blood occurs during a bleeding, the later samples being more dilute than the earlier.

These results, however, may not apply to blood *in vivo*, and attempts were made to estimate the urea in plasma obtained by centrifuging a ligatured vein (Van Creveld and Brinkman, 1921). The difficulties of estimation due to the small quantities of fluid obtained have so far prevented conclusive results being reached, but the figures, as far as they go, lend no support to the view that urea is exclusively present in the plasma.

In order to remove all uncertainty regarding the blood concentrations available for secretion, it was concluded that the estimations in the concentration ratio experiments had best be done on plasma; a few which were performed on whole blood, in which the assumption was made that there was equal distribution, gave results which agreed very well with the former.

SUMMARY.

(1) Urea and creatinine are evenly distributed between plasma and corpuscles in the blood of the cat.

(2) After the injection of urea and creatinine into the blood-stream the substances may be found equally distributed between plasma and corpuscles, or there may be a greater proportion in the plasma.

(3) After the injection of inorganic phosphate and uric acid a larger proportion is found in the plasma relative to the proportion present in the corpuscles.

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VASCULAR CHANGES AFFECTING BULBAR NERVE CENTRES.

D. T. BARRY.

From the Institut Marey, Boulogne-sur-Seine, and the Physiological Laboratory, University College, Cork.

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CHANGES in the supply and hæmoglobin content of the blood cause variations in tone and excitability of bulbar centres which do not seem to have been systematically studied, though mentioned in the work of Bayliss (1920). The present work represents an attempt to investigate these effects in the dog.

METHODS.*

The right vagus only is cut. Stimulation of the cut nerve (central end) induces slowing of the heart with or without vaso-motor effects. Most cases chosen gave pure inhibitor response. The first record (Fig. 1) shows the type of reaction selected to work upon. The fall in blood-pressure is a cardio-inhibitor effect, for blocking of the left vagus stops it. Plethysmograph records of a limb also show that it is not a vaso-dilator effect. The strength of stimulus for eliciting this response varied, but was uniform for each series of observations. The blocking current, unless very strong, does not excite the vagus. With an ascending current from a cell with one volt or so difference of potential, blocking is complete on closure and recovery immediate on opening; the effect is the same if the current be reversed (kathode below).

The anæsthetic used was ether or C.E. mixture after a preliminary dose of morphia (6 mgr. per kg.). The anæsthetic was given either by artificial respiration by a trachea tube, or by natural respiration. The degree of anæsthesia was as uniform as possible throughout. The chief disturbing factor was change in the breathing. Occasionally artificial respiration was used to help revival. Measured quantities of blood were drawn by a cannula in the femoral artery. This was in some instances supplemented by clipping one carotid, the other carotid being connected with a manometer (Fig. 2). Reintroduction of the whipped blood by the jugular vein, as well as that of gum saline, was also studied.

Acting on the advice of Sir William Bayliss, to whom I am greatly indebted, I have used a 6·5 per cent. gum solution in all the later experiments, though at first only a little more than 5 per cent. was used, which had a viscosity between that of blood and that of serum. The 6·5 per cent. with its higher osmotic pressure and higher viscosity tends to maintain better a given pressure level.

It is believed that the tone of the cardio-inhibitor centre is depressed by hæmorrhage, and the warning to avoid this (Bainbridge, 1914) if one wishes to elicit facile reflex responses implies that this facility is a characteristic of tone, and may suggest that excitability and tone are synonymous. McWilliam (1900) in his work on chloroform effects on nerve centres discriminated between the two properties. Tone may be induced or increased reflexly in the cardio-inhibitor centre, *e.g.* by stimulating the central end of the vagus, as it may be depressed, *e.g.* by somatic nerves. The degree and duration of these artificially induced effects, as well as the natural state of tone, are taken in these experiments in conjunction with the immediate results of reflex excitation as a measure of the functional state of the centre under different conditions. They are considered under two heads—immediate and remote (see protocols). The duration of an effect indicates any changes of tonic activity in the cardio-inhibitor centre, while the promptness of the response and its extent (change of heart-rate) give an idea of reflex excitability. Blocking and unblocking also reveals the tonic condition.

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* Abbreviated at the request of the editors.

For investigating the effects of small hæmorrhages most cases chosen yielded a fairly well-marked slowing reaction to vagal stimulation (Fig. 3), and change of rate rather than of pressure has been the criterion—that is to say, that other centres are so slightly affected by small hæmorrhages as not to be worth investigation. When amounts of 50 per cent. or more of the total blood (on basis of 7 per cent. of body-weight) were rapidly bled, the effects were studied on cardio-inhibitory, vaso-motor and respiratory centres. Vaso-motor tone is measured by rise of pressure occurring spontaneously after block, and excitability by increased rise on stimulation of central end of vagus and sciatic.

A depressor vaso-motor effect has been rarely observed in these dog experiments to result from central vagus stimulation as described. Changes in the pressor response and tone are taken as the chief indication of the effects of bleeding on the vaso-motor centre. In testing the activity of the respiratory centre sciatic and vagus stimulation was employed. To the former the centre responds by strong inspiratory efforts.

RESULTS.

The protocols of some experiments are given, so as to reveal the method at a glance and to give the more important reactions. The procedure adopted is set out in the order of performance. Exp. III represents an exhaustive method carried out in some instances with extreme bleeding in a series of steps, re-bleeding after gum saline, and the stages of recovery with gum saline and blood contrasted. Before infusion in such cases the heart usually came to a stop, or showed extreme slowness and irregularity (Fig. 7) at or about the zero line of pressure. The amount of blood which could be drawn off before reaching this level is referred to as "the yield." The proportion of this amount to the calculated total varied from 60 to 85 per cent., according to rate of bleeding, condition, etc.

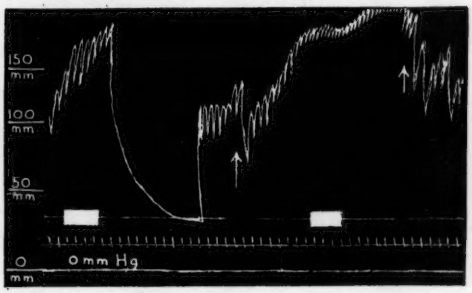

FIG. 1.—Well-marked reflex from cardio-inhibitor centre. It is annulled by blocking left vagus (first arrow). Sustained tonic activity shown when this is unblocked (second arrow). (Block by continuous current with difference of potential of about 1 volt.)

Jonescu (1900), with Bayliss, investigated the effects of asphyxia on the secretory powers of the salivary glands deprived of blood or oxygen, and noted a rapid decrease in secretion; the limits of the privation period consistent with recovery were not fully established. In the reaction from nerve centres investigated in the present experiments we possess a reliable key to tissue activity. The response is almost invariably the same to a given stimulus in uniform conditions over long periods. The rapidity is a great advantage as compared with secretion reaction as a test of tissue condition, that is, the condition resulting from mere blood changes on the one hand, and, to some slight extent, from the secondarily induced shock accompanying anæmia on the other.

Dolley (1909) has shown that nerve cells undergo morphological change from deprivation of blood, but in that case the duration period was not determined for the abolition of function.

EXPERIMENT I.—*Weight of Dog, 6 kg. Yield of Blood, 320 c.c.*

Nerve stimulation.	Amount bled.	Amount infused.	Left vagus.	Immediate effect of stimulus.	Latent period.	Heart rate.	Later rate.	Respiration.
	c.c.	c.c.			Secs.			
C.E.V.	—	—	Free	Fall B.P. and slowing	1·5	120–20	60	Inhib.
,,	150	—	,,	Ditto	2·5	120–50	100	,,
Sc.	—	—	,,	Rise B.P. accln.	1·5	100–130	100	Inc. rate and depth.
C.E.V.	150	—	,,	*Nil*	—	—	18 irreg.	Expy. efforts.
Sc.	—	—	,,	Slight rise	2	*Nil*	Irreg.	,, ,,
C.E.V.	—	150 g.s.	,,	Rise B.P., later slowing	1·5 for rise	110–80	100	Inhib.
,,	—	150 g.s.	,,	Slowing	1·5	110–80	100	,,
,,	200	—	,,	*Nil*	—	—	120	Slight inhib.
,,	—	300 blood	,,	Slowing	1	120–60	70	Inhib.
,,	—	—	Blocked	Rise B.P.	1·5	—	120	,,

EXPERIMENT II.—*Weight of Dog, 14 kg. Yield of Blood, 875 c.c.*

C.E.V.	—	—	Free	Slowing fall B.P.	1	150–0	100	Inhib.
,,	275	—	,,	,, ,,	1·5	200–0	150	,,
,,	150	—	,,	,, ,,	2	150–50	120	,,
Sc.	—	—	,,	Rise B.P. accel.	1·5	150–180	180	Inc. rate depth.
C.E.V.	100	—	,,	Slowing	3	180–100	140	Inhib.
,,	150	—	,,	Rise B.P.	1·5	*Nil*	150	,,
,,	150	—	,,	*Nil*	—	,,	Irreg.	,,
,,	—	—	Blocked	,,	—	—	Irreg.	Slight inhib.
,,	50	—	Free	,,	—	—	Stopped	Expy. efforts stop in expn.
,,	—	700 g.s.	,,	Slowing	2·5	140–10	50	Inhib. after recovery.
,,	—	—	Blocked	Rise B.P.	2	*Nil*	120	Inhib.

EXPERIMENT III.—*Weight of Dog, 8 kg. Yield of Blood, 500 c.c.*

C.E.V.	—	—	Free	Slowing fall B.P.	1·5	120–60	80	Inhib.
,,	150	—	,,	,, ,,	2	140–80	130	,,
,,	50	—	,,	,, ,,	2	150–100	120	,,
,,	50	—	,,	,, ,,	2·5	180–160	170	,,
,,	100	—	,,	Falls B.P.	—	*Nil*	160	,,
,,	—	—	Blocked	Rise B.P.	2	,,	160	,,
,,	100	—	Free	*Nil*	—	,,	100	*Nil.*
Sc.	—	—	,,	,,	—	,,	100	Slight increase.
,,	—	—	Blocked	,,	—	—	100	,, ,,
C.E.V.	—	—	,,	,,	—	—	—	Inhib. slight.
,,	50	—	Free	*Nil* (zero pressure)	—	—	*Nil*	Expy. stop *nil.*
,,	—	170 g.s.	,,	*Nil* (20 mm. Hg.)	—	—	100 irreg.	*Nil* (stopped).
,,	—	150 g.s.	,,	Slowing	4	120–100	120	Inhib.
,,	—	200 g.s.	,,	,,	2	150–90	130	,,
Sc.	—	—	,,	Rise B.P.	2	130–150	150	Increased.
,,	450 (2nd Bleeding)	—	,,	*Nil*	—	—	*Nil*	Stop slight exp.
C.E.V.	—	400 blood	,,	Slowing	1·5	130–60	80	Inhib.
,,	—	—	Blocked	Rise B.P. accel.	1·5	120–140	100	,,
Sc.	—	—	Free	Rise B.P.	2	*Nil*	120	Increased.

We may consider the results in relation to each of the three centres:

(1) *Cardio-inhibitor.*—The animals included in the Table gave a more or less marked slowing of beat as a response when the central end of the cut vagus was stimulated. It will be noticed that bleeding up to 30 or 40 per cent. of the total yield, and up to 50 per cent. of the calculated total, leaves the reflex response but slightly affected, though it disappears generally when this limit is passed. Gesell (1918) found in some cases, for the respiratory and CO_2 curves, great resistance up to a certain degree of hæmorrhage, and then a sudden break. The loss of cardio-inhibitor reflexes is, as a rule, sudden, rarely progressive. The recovery process after infusion is more progressive. Occluding the carotid artery is a much more effective procedure in suppressing the inhibitory reflex than is bleeding of moderate degree (Fig. 2). Effects frequently noted from moderate bleeding were the lengthening of the latent period (Fig. 3) of the reflex inhibitory response and the diminution of its duration; the latent period decreases again with recovery (Fig. 4). Activity of the cardio-inhibitor centre does not depend on a given arterial pressure; in slow bleeding responses may persist down to a pressure of

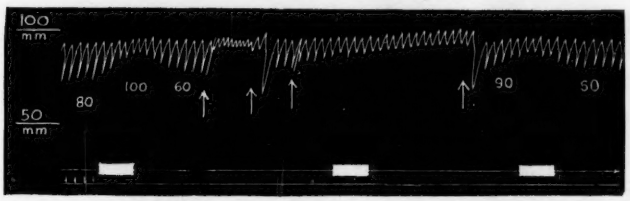

FIG. 2.—Inhibitor reflex of slight degree abolished by occlusion of carotid artery between second pair of arrows and return of it when the artery is free.

50 mm. Hg. or less, and even if abolished may return again at low pressures. Clipping the carotid artery interferes greatly with the blood supply, and the cardio-inhibitor centre fails quickly; yet the vaso-motor centre maintains a considerable degree of activity under this condition. It thus affords a contrast between them. There is no important disturbance of bulbar circulation by occlusion of one carotid and one jugular. This has been remarked already (Roberts, 1921). Respiration, Roberts says, is not influenced by clipping the artery of the other side in addition, the only result of this being a slight rise, of mechanical origin, in the blood-pressure. But a disturbance of bulbar reactions results from this occlusion. The most obvious of them is the failure of (a previously well-marked) response of the cardio-inhibitor centre; this change I have found to be invariable; there is also increased heart rate, which shows diminished tone of the centre.

Gesell (1918) concluded that a great reduction of volume flow may occur with a constant head of pressure; this flow may, with change of pressure, remain constant or change in either direction. As seen by the uniform reactions of bulbar centres, especially the cardio-inhibitor, the flow to them seems to remain uniform, or at any rate efficient, at low pressures, except after rapid bleeding, when it is disturbed for a time. It is also disturbed by

clipping the second carotid. The reduction in heart-rate from central vagus stimulation after withdrawal of 40 per cent. of the calculated total blood (300 c.c. in an animal of 10 kilos), and even up to 70 per cent., sometimes may be nearly as great as prior to bleeding, yet the moment the artery is clipped the response is abolished. The cutting off of the direct supply by the carotid evidently greatly hampers the circulation in the centre, even though both vertebrals are free. The duration of arterial occlusion also affects that of the reflexly induced tone in the centre. After removal of the clips the pulse-rate tends to be re-established rapidly when stimulation ceases, though the reflex response of slowing may have been immediate. It shows distinctly the difference between the properties of reflex excitability and tonic activity as affected by oxygen want. Bleeding first affects the latent period and the duration of reflex tone.

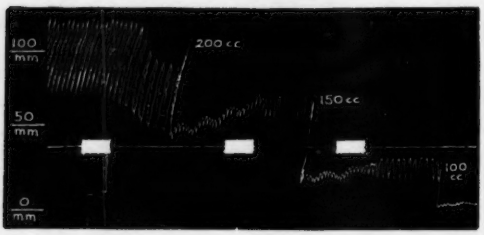

FIG. 3.—Prolongation of latent period of cardio-inhibitor reflex after bleeding.

The withdrawal of small amounts of blood such as may represent that lost in hurried dissection has little effect in altering the condition of the cardio-inhibitor centre. A more probable cause of such alteration is rough handling of the tissues. I have frequently noticed that when the carotid sheath was stretched, dragged upon and torn rapidly open, the responses of the centre, either to vagus or sciatic stimulation, were not well marked. Shock of this sort is distinctly deleterious to the centres. When the sheath is dissected with care and the general handling of the tissues is gentle, the reflex excitability of the vagus centre and the facility of inducing tone in it are much better for a given degree of anæsthesia. In some instances it was noticed that the latent period of the slowing was very long (5 to 10 seconds) even before bleeding; moderate bleeding more quickly abolishes the reflex in such cases (Fig. 5). Bayliss (1918) and others ascribe the chief symptoms of shock to absorption from tissues injured, but do not altogether discard reflex effects on the centres. I think this latter factor comes in, at any rate in neck dissection with tissue abuse, in affecting the reactions of bulbar centres. Owing to restriction of space only a few records illustrating the protocols are inserted, but the more important results are explained by these. Fig. 4 represents a condition reached as follows: The animal (8 kg.) was bled to the extent of 600 c.c.; gum saline infusion was carried out and recovery of the reflex slowing with the standard stimulus occurred. The animal was again bled. The heart had now become very slow

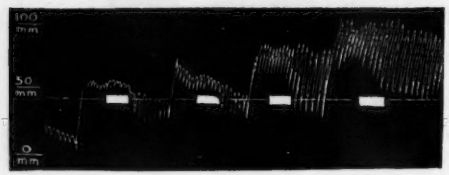

FIG. 4.—Recovery of inhibitor reflex with gradual shortening of the latent period. 100 c.c. blood infused at each stop. Dog 10 kg.

and irregular after the second bleeding, and a little more gum saline was introduced which brought it to the condition of the first step shown in Fig. 4 (B.P. about 15 mm. Hg.) before infusion with blood. At this stage the C.E.V. stimulation gave no response; as blood was infused the response reappeared and improved rapidly—more rapidly than with gum saline. The restorative effect of gum saline, when the heart is in very poor condition and the centres are exhausted, is not quite so good as that of blood. Fig. 6 shows practically no recovery of the inhibitor response with gum saline in such a condition. Fig. 7 is from a similar instance (high yield and secondary bleeding); gum saline is not equal to blood in effecting recovery either of tone or excitability. The necessary hæmoglobin being added, in this case recovery was prompt. The privation of the centres was extreme and the hæmoglobin limit passed. It will be referred to again under vaso-motor effects.

(2) *Vaso-motor.*—In most cases, after full yield of blood, all three centres showed good recovery with gum saline alone, but when one centre failed in such circumstances it was generally the vaso-motor. Vitality, although without regularity, may still be shown by the respiratory centre, though perhaps without possibility of ultimate full recovery. The vaso-motor centre is judged to fail by reason of loss, not of reflex response, but of tone. Blood sometimes remedies this failure when gum saline cannot, when the yield has been very high. The rise of pressure after clipping the second carotid artery seems to me to be somewhat greater than that attributable to mere mechanical effects. There is a small but continued rise, and when the clip is removed the sudden drop is greater than the immediate rise on putting it on. After moderate bleeding there is also a steady rise of pressure. The centre becomes more active either as a result of volume change or oxygen want. It is also more responsive to reflex excitation in these circumstances. The vaso-motor centre seems to be excited by the disturbance of the clip in the opposite direction to the effect on the inhibitor centre. The vaso-motor centre remains active—shows tone and excitability—for some time after the cardio-inhibitor properties are lost in progressive bleeding. Its tonic functions are quickly suppressed at low pressures before excitability goes—the centre can respond to a stimulus but the effect passes off quickly.

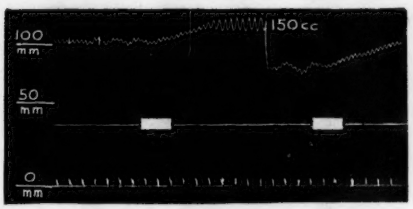

FIG. 5.—Inhibitor reflex with long latent period abolished by moderate bleeding—20 per cent. of total.

Bayliss has remarked that it is useless to employ transfusion or gum infusion if the vaso-motor centre vitality be lost. His observation that it is impossible, in such a condition, to raise the pressure beyond a low level, except temporarily, even by injection of large volumes, has been verified in these experiments (Fig. 6). The record shows in the different steps of gum saline infusion that there is no response from the centres. There had been some half hour's exposure to low gum saline pressure, but in a poor type of animal. The pressure rose to about 100 mm. Hg. as a maximum, but it fell away

quickly when infusion ceased. Blood was immediately tried to replace gum saline, but with no better result. But in other cases of severe bleeding blood was more effective than gum. In similar cases where the recovery of pressure likewise failed with gum saline, the cardio-inhibitor centre was shown to be still active—reflexly excitable and active in tone (Fig. 8). The survival powers of vaso-motor and cardio-inhibitor centres present somewhat anomalous relations. In several instances the latter reacted when the former remained inactive. The cardio-inhibitor response goes more rapidly than the vaso-motor, in rapid bleeding, but with gum saline infusion it returns more readily than the vaso-motor response. In Fig. 8 the cardio-inhibitor centre responds well to stimulation but the vaso-motor centre does not. In all the animals whose blood-pressure was easy to restore there was generally a ready return of reflex response in both centres. Death of the vaso-motor centre means failure of the necessary circulation pressure, except for brief periods. A remnant of vaso-motor vitality, on the contrary, means recovery if the respiratory centre be not too far gone; but this recovery is often not full, whereas the

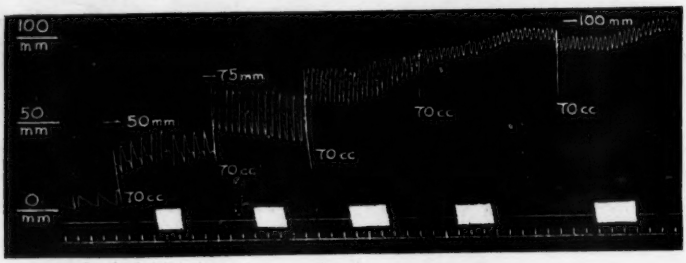

FIG. 6.—Suppression of function in both vaso-motor and cardio-inhibitor centres after severe bleeding and exposure. Infusion of gum saline.

cardio-inhibitor recovery is frequently so. Vitality of the vaso-motor centre is apparent when the reflex response is recovered, but a loss of reflex does not mean death, though the margin between the excitability threshold and absolute failure seems to be small. It sometimes recovers late (Fig. 9, end of tracing). With the cardio-inhibitor centre, on the other hand, reflex excitability and tone, as elicited through the vagus, may be suppressed over long periods and yet the centre may show eventual recovery. Its reflex reactions appear to succumb more quickly than those of the vaso-motor centre, but its vitality and resistance are really greater (Fig. 8). In some instances, if bleeding be slow, quite a good response is given by the cardio-inhibitor centre down to pressures of 30 to 40 mm. Hg. These cases recover very well with gum saline, the vaso-motor and respiratory centre also often recovering if exposure to low pressures has not been too prolonged. In Fig. 8 there is every sign of death of the vaso-motor centre—no response to sciatic or vagus excitation and no tone—but the inhibitor centre is quite active.

We sometimes see a difference between the effects of gum saline and blood as restoratives; with equal amounts a lower pressure is established by the former, though restoration of reflex function, both in vaso-motor

and cardio-inhibitor centres, may be quite good. The vaso-motor centre, however, must be defective in tone in such cases, though its excitability be good. The all-important factor, as Bayliss (1918) points out, is the oxygen carrier, and it is certainly so, for tonic and permanent recovery. The tonic activity of the centres seems to depend more on a due supply of oxygen than the reflex excitability, which may still be exhibited in pronounced oxygen want. Blood infusion or transfusion is the desideratum in cases of severe hæmorrhage in human beings, but gum saline, while effective as a permanent agent in cases with a sufficient hæmoglobin remnant, should not be lost sight of as a temporary expedient in still more serious loss.

(3) *Respiratory.*—Roberts (1921) says the respiration ceases on sudden free hæmorrhage from the aorta, because of anæmia of the medulla. Anæmia of the medulla brought about slowly in the dog does not always stop respiration. For instance, in an animal of 6 kilos 320 c.c. were drawn off in lots of 70 to 80 c.c., at intervals of two to three minutes; the respirations, after some increase of rate in the early stages of bleeding, were reduced to 4 or 5 per minute, but remained regular. They increased to 10 per minute after gum saline. Roberts got full stoppage of respiration by clamping all four arteries to the brain, but, with a supply from one vessel only, the respirations remained quite frequent and regular. The respiratory centre resists change in volume flow very well up to a certain point. Death of the centre, as a rule, quickly supervenes once this point is passed.

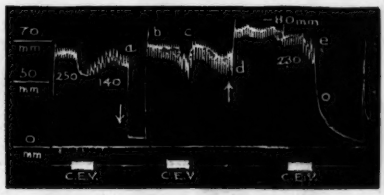

FIG. 7.—Bleeding and re-bleeding; second "bleeding" of gum saline and reintroduction of gum saline shown at *a* and *b*. There is no inhibitory response. But at *d* some 150 c.c. of blood were run in and the cardio-inhibitory centre recovered function (third signal).

The respiratory centre responds well to sciatic stimulation to the very end. When near exhaustion this response may take on an active expiratory character. This expiratory response is also usually elicited at this stage by stimulation of the central end of the vagus, which normally causes apnœa with all strengths of current. In this condition, too, even the weak blocking current sets up some expiratory efforts, the expiratory part of the centre being evidently very sensitive.

Doubt still exists as to the relation between the respiratory and cardio-inhibitor centres. In these experiments no overflow was found from the former to the latter; this confirms the observations of Bainbridge (1920). In some dogs respiration is normally very slow. Even morphia, which generally causes panting, causes little or no increase of breathing in these, and the centre may display a great amount of resistance, and is especially prone to exhibit the expiratory response above referred to.

Bayliss (1920) has drawn attention to the fact that the respiratory is more sensitive than the vaso-motor centre. But the respiratory centre may be active, with gasping respirations, or even regular breathing, at a time when all response is abolished in the vaso-motor and cardio-inhibitor centres—for instance in profuse rapid bleeding with or without administration of gum saline.

Bayliss considered the gasping condition of the respiratory centre as a state or sign of failure in which the heart is insufficiently supplied with oxygen. That is certainly so. The present work merely shows that by prompt action the centre may be restored from an apparently hopeless condition. In ultimate survival, which evidently Bayliss means, it is different in this respect: the respiratory is not at all so resistant as the cardio-inhibitor centre. While its reflex or automatic activity may be exhibited when all other reflexes and tonic activity are suppressed, and when it is in a dying condition, it may fail just as these are being revived in the cardio-inhibitor centre; and it may fail even when the vaso-motor centre is active (this requiring artificial respiration for its demonstration), but in a majority of the present experiments the vaso-motor centre died before the respiratory centre. For that reason alone I place the vitality of the respiratory centre as somewhat higher than that of the vaso-motor centre.

The respiratory centre, having become inactive from oxygen want, cannot recover with gum-saline alone, although the heart beats, the inhibitor centre responds and a pressure of 70 or 80 mm. Hg. is reached. In this condition I have frequently brought about recovery of the respiratory centre (Fig. 10) by the addition of blood to the extent of 10 per cent. of corpuscles. The corpuscles were measured roughly by centrifuge (1/10) and more accurately with hæmocytometer (1,000,000 to 1,100,000 per c.mm.), as there was none of the clumping described by Bayliss (1920) for cats. In some of these cases there was no restoration of function in the vaso-motor centre. Prolonged expiratory efforts usually mark the close of activity in the respiratory centre. When the centre is failing a sciatic stimulus remains effective in producing the well-known increase in activity which affects both phases, but, as usual, chiefly the inspiratory one. A rise of blood-pressure accompanies this increased ventilation, which is in part mechanical and in part due to a feeble response of the vaso-motor centre. In most instances the mechanical effect alone occurs as the vaso-motor centre is not responsive.

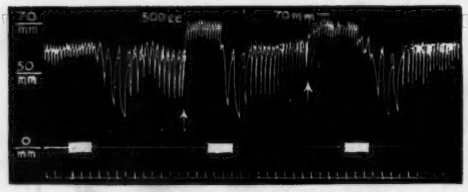

FIG. 8.—Good recovery shown in the inhibitor centre with complete failure of the vaso-motor centre. Natural respiration. The pressure never rose beyond 70 mm. Hg. and there was no reflex rise on sciatic stimulation—(not shown).

DISCUSSION OF RESULTS.

In placing the cardio-inhibitor centre first in order of temporary failure, account is taken of the increased length of the latent period, the well-marked effect of clipping the artery, the diminished tone effect and that of sudden bleeding. The order of suppression (temporary) of function in these bulbar centres is different from that of their irretrievable abolition. Thus:

Temporary suppression.	*Abolition.*
(1) Cardio-inhibitor.	(1) Vaso-motor.
(2) Vaso-motor.	(2) Respiratory.
(3) Respiratory.	(3) Cardio-inhibitor.

The order means that while the respiratory centre holds out after function has been suppressed in the other two, yet it is more liable to suffer permanent damage than the cardio-inhibitor centre, if we take into account their recuperative powers. The relative positions of the vaso-motor and respiratory centres are somewhat doubtful. In some cases the vaso-motor centre resists apparently better than the respiratory, but, since in so many experiments the former succumbed first, I think it is the more vulnerable. As the respiratory centre is failing, it becomes less responsive to the inhibition produced by vagus stimulus, though even more sensitive to sciatic stimulation, which produces increased inspiratory efforts. One notes the diminished vagal inhibitory response at a time when the cardio-inhibitor centre is still quite active. The centre later still shows the expiratory type of response, already mentioned, to vagus stimulation. The increased CO_2 in the blood with the accompanying diminution of pH makes the centre extremely sensitive, as pointed out by Haldane and Priestly (1905). This increased sensitiveness connotes a greater tendency to escape from inhibition. There is practically no response in the dog upon which one may count with more certainty than that of respiratory inhibition to central vagus stimulation, of all degrees of intensity, but it becomes diminished as the sensitiveness of the centre increases in another direction. In the dog respiration is generally slow (12 to 15 per minute) when the panting has passed off after a morphia injection. In the early stages of hæmorrhage it increases in rate slightly; later, at a time when the centre is very sensitive, it becomes very deep, and is gradually reduced in rate in further privation. As Bayliss (1918) points out, quick and shallow respiration does not result in more effective ventilation. I have never seen the rapid shallow respiration described as accompanying some forms of shock and acidosis in these cases of simple bleeding. Bayliss explains the shallow breathing by a modification of the Hering-Breuer reflex in which the inhibitory response of the centre would be exaggerated. The respiratory centre in the cat shows some difference from that of the dog, *e. g.* in reacting to a tracheal plug; this is possibly another. The inhibitor effect of C.E.V. stimulation is certainly diminished in the dog with failing centre when it seems to be increased in the cat.

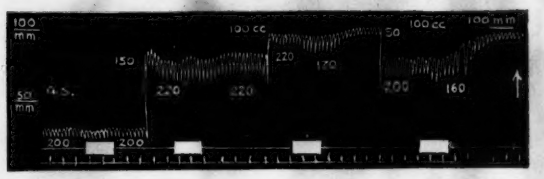

FIG. 9.—Slow recovery of cardio-inhibitory centre functions. Vaso-motor centre (tested at stops) functionless till near the end. Note that the pressure has actually fallen with last portion of the infusion.

In reference to the superiority of blood over gum saline in recovery, it must not be taken as contradicting the contention of Bayliss (1922[1]), that gum saline was quite as effective as blood. He always assumes that sufficient hæmoglobin is left to carry on oxygenation, whereas in most of my experiments where the opposite is claimed, that reserve was passed. The quick return of the inhibitor response (Fig. 7) is a case in point. The necessary hæmoglobin here quickly effected what the gum saline failed to do. Though I have some-

times seen an apparently quicker recovery of function with blood, for less severe bleeding, yet in general, as Bayliss remarks, it is surprising what gum saline can do in presence of small amounts of hæmoglobin. Where it is simply a question of increasing the volume of fluid to the tissues, then if the hæmoglobin content is adequate, gum saline serves as well as blood for the overcoming of stasis and reduced alkaline reserve resulting from deficient oxidation, and must be considered an invaluable agent in such cases. Where the hæmoglobin content is defective, gum saline can only produce a temporary though sometimes striking return of activity in the centres. Gum, I think, hastens clotting. I have not seen this effect referred to. It seems a salutary one, though very troublesome in experimental work of this sort. Sir William Bayliss suggests to me that it may be due to the calcium of the gum.

The total amount of blood which may be removed, before the pressure falls to the zero line and the heart-beat fails, is variable. Sometimes the heart

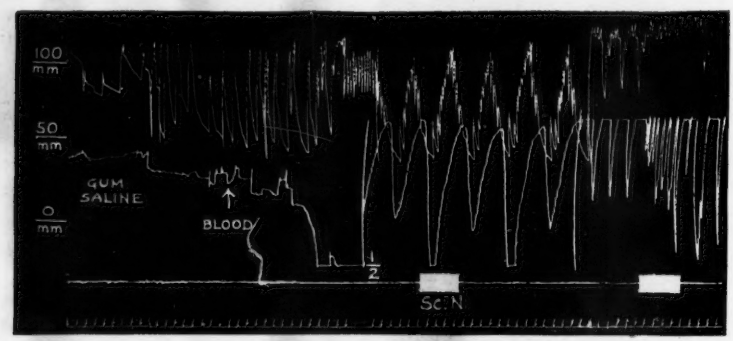

FIG. 10.—Failure of respiratory centre with gum saline (20 minutes at low pressure). Return of activity with blood (begun at arrow). Delayed return of reflex to sciatic stimulation (second signal). No response, first signal. Upper tracing = blood-pressure; lower tracing = respiration; down-stroke represents inspiration.

becomes irregular after a loss of 60 per cent. of the calculated total, if bled rapidly; with slow bleeding, in steps, the loss may proceed to 80 per cent., or more, of the total. The condition of the animal and the time that it has been under experiment also affect the result. Animals rapidly bled to a state of great slowness and irregularity of the heart show good recovery with gum saline infusion if not delayed.

Bayliss ascertained the time-limit for recovery of bulbar centres in cats exposed to low blood-pressure of 58–60 mm. Hg. to be as long as 1½ hours or more. In the present work the time-limit was investigated in the case of still lower pressures than those employed by Bayliss. After full yield of blood to heart-stop and zero-pressure a little gum saline was introduced to keep the heart going at some 30 mm. Hg., and it was found that the centres sometimes recovered after half an hour under these conditions. This corresponds fairly well with the results of Richet and his associates (1918) for recovery in dogs. Stewart, Guthrie, Burns and Pike (1906), who deprived the medulla of blood by occlusion of vessels, found varying intervals for recovery after complete anæmia.

They considered half an hour exceptionally long. The condition so produced must differ in some important respects from that due to hæmorrhage.

Full recovery in the respiratory and vaso-motor centres after more than fifteen or twenty minutes was rare. There is a difference in the efficiency of a pure gum saline solution and the same plus a little blood in the survival of the starved centres in such circumstances. Athanasiu and I (1922), working at the Marey Institute, found that survival in the nerve centres of dogs, as exhibited by the electrical variations, occurred with artificial circulation from the heart-lung preparation of a second dog some twenty minutes after the centres had been drained of blood, and after more than an hour of shock from the very severe operation of exposing cerebrum, medulla, cord and nerves. Prior to the irrigation the animal was fully bled.

The duration of the severe anæmia consistent with good recovery after total bleeding does not exceed half an hour for the vaso-motor and inhibitor centres, more for the latter than the former. If the time were longer than this, recovery in vaso-motor and respiratory centres did not take place at all, and the heart-beat, with re-establishment of low pressures, recovered only temporarily; in some of these only the cardio-inhibitor centre remained responsive. The respiratory centre and the heart-beat, not the pressure, afford the indications for the amount of gum saline to be employed during the privation period. Both keep active sometimes at very low pressures which maintain a fixed level with or without a little extra gum saline. Complete privation, that is, when heart-stop and zero pressure result, cannot be supported for more than a few minutes. But even in such cases, if the yield is small, the heart may resume its beat spontaneously, and in these it is only by secondary bleeding, after the introduction of some gum saline, that one can reach a very low blood content.

Bayliss (1922[2]) has calculated for cats that 70 per cent. of the total blood is replaceable by gum saline, consistently with complete recovery. The fluid then would contain about 12 volumes per cent. of corpuscles. Henderson and Haggard (1922) removed 80 per cent. of the blood (on a basis of 7·3 per cent.) and got recovery with gum. Bayliss (1922[2]) thinks their percentage must have been larger, *i.e.* working on a lower basis. In my experiments there is no question of lasting recovery, but only of a temporary restoration of function, with gum saline. More than 70 per cent. of blood was removed, on whatever basis we work, yet function in nerve-centres was re-established with gum saline. The degree of hæmorrhage compatible with recovery of function was extreme, but accurate measurements of the duration of this privation period could not be made. Certainly, in some instances, such as that of No. 2, a period of half an hour elapsed from the end of a second "bleeding" to the beginning of infusion. The fluid that came away in the second bleeding was generally but light red in colour, and contained 7 or 8 per cent. by volume of corpuscles.

CONCLUSIONS.

The reflex excitability of bulbar nerve centres differs from their tonic activity, whether natural or induced by reflex excitation. Bleeding up to 40 or 50 per cent. of the calculated total blood affects the first-mentioned less than the second, though both properties depend on a proper blood supply. The vaso-

motor centre is less rapidly affected by change of volume flow and oxygen content than is the cardio-inhibitor centre. Both function after excessive bleeding and infusion of gum saline, until the hæmoglobin is reduced to about one-fifth of the normal amount (80 per cent. of the total blood removed). Loss of blood amounting to 85 or 90 per cent. and brought about by secondary bleeding was not sufficient to cause death of the centres, if not too long exposed to these conditions. The vaso-motor centre in these extreme conditions is the most susceptible, though there is little difference between it and the respiratory centre. The cardio-inhibitor centre, though the earliest to suffer, is the most resistant to irreparable damage.

Rough handling of the tissues in dissection is a more serious cause of disturbance of reflex responses in the nerve centres than moderate amount of hæmorrhage.

Gum saline effects recovery of the centres after long periods of exposure to very low hæmoglobin content, represented by about 7 or 8 per cent. by volume of corpuscles. For permanent recovery blood is needed in addition for these extreme cases; in lesser degrees of privation, to 12 to 15 per cent. of corpuscles, gum saline alone rapidly effects recovery.

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